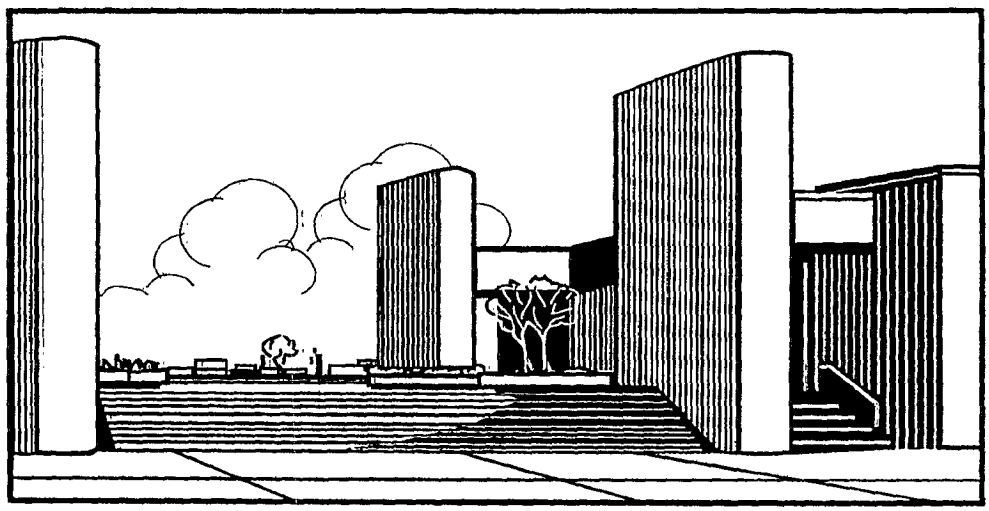
TECHNICAL/COST PROPOSAL

PROVIDE INHALATION TOXICITY STUDIES OF CLIENT'S PROPRIETARY PRODUCT BNW No. 20643

In Response to Letter Request

PHILIP MORRIS

September 15, 1993



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NTP 25% due to National Lab System

Vapor studies 2 species 3 dose 50 / sex / sp

< 350K - generation & mainte

750K subchronic

150 K

$4 M chronic

lung clearance, burden

>

Whole body

Chemistry

equip. setup

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Battelle
Pacific Northwest Laboratories
Battelle Boulevard
P.O. Box 999
Richland, Washington 99352
Telephone (509)

September 15, 1993

Dr. George J. Patskan
Philip Morris
Research Center
P. O. Box 26583
Richmond, VA 23261

Reference: Your letters to Dr. Bernie Greenspan dated October 20 and November 19, 1992

SUBJECT: PROVIDE INHALATION TOXICITY STUDIES OF CLIENT'S PROPRIETARY PRODUCT
BNW No. 20643

Dear Dr. Patskan:

Battelle-Northwest is pleased to submit six copies of the accompanying Technical/Cost Proposal in response to the above referenced letter.

Battelle-Northwest proposes to conduct the research directed toward the objectives outlined in the accompanying proposal on a cost-reimbursement, best-efforts basis within the available time and funds under the terms of any resulting contract. All of the terms and conditions, including the scope of the proposed research, will be subject to mutual agreement.

It is anticipated that a small portion of the chemistry research and development may be conducted in part, in a U. S. Department of Energy facility. Therefore, certain terms and conditions of the Use Permit Contract between the U. S. Department of Energy and Battelle-Northwest, indicated on the enclosed "Provisions for Contracts Utilizing Government-Owned Facilities and Equipment, or Certain Unreported U. S. Department of Energy Information," must be incorporated in the resulting agreement.

If you have any questions about our proposal, please direct those of a technical nature to Dr. Earl W. Morgan on (509) 375-6318. Contractual matters should be addressed to Mr. Kirk Heaton, Contract Associate, on (509) 376-1336.

We look forward to being of service to Phillip Morris, and to hearing from you regarding our proposal.

Very truly yours,

Cheryl L. Nickola
Contracting Officer

CLN:nj

Enclosures

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TECHNICAL PROPOSAL

CARCINOGENICITY STUDY OF ROOM-AGED SIDESTREAM CIGARETTE SMOKE IN MICE

PROJECT NO. 20643

In Response to Letter Request

from

Philip Morris
Research Center
P.O. Box 26583
Richmond, VA 23261

September 1993

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BATTELLE MEMORIAL INSTITUTE
PACIFIC NORTHWEST LABORATORIES
P.O. Box 999
Richland, Washington 99352

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LIST OF ABBREVIATIONS

AAALAC	=	American Association for the Accreditation of Laboratory Animal Care
AALAS	=	American Association of Laboratory Animal Science
ABI	=	Applied Biosystems, Inc.
ACVP	=	American College of Veterinary Pathologists
AES	=	Atomic emission spectroscopy
ALS	=	Automatic liquid sample
APCI	=	Atmospheric pressure chemical ionization
ASCP	=	American Society of Clinical Pathologists
ASQC	=	American Society of Quality Control
ASTM	=	American Society for Testing and Materials
AU	=	Absorbance units
BCL	=	Battelle Columbus Laboratories
BNW	=	Battelle, Pacific Northwest Laboratories (Battelle-Northwest)
bp	=	Base pair
CBC	=	Complete blood count
CI	=	Chemical ionization
CO	=	Carbon monoxide
CO_2	=	Carbon dioxide
COHb	=	Carboxyhemoglobin
DBCP	=	Dibromodichloropropane
DC	=	Direct current
DEE	=	Diesel engine exhaust
DENA	=	N-Nitrosodiethylamine
DETBA	=	Diethylthiobarbituric acid
DMNA	=	N-Nitrosodimethylamine
DNP	=	Dinitropyrene
DNPH	=	2,4-Dinitrophenylhydrazine
DOE	=	U.S. Department of Energy
EAFS	=	Ethanol/acetic acid/formaldehyde/saline
EPA	=	U.S. Environmental Protection Agency
ES&H	=	Environmental, Safety, and Health
ETS	=	Environmental tobacco smoke
FDA	=	Food and Drug Administration
FTIR	=	Fourier transform infrared detection
GC	=	Gas chromatography
GDVII	=	Theiler's murine encephalomyelitis virus
GLP	=	Good Laboratory Practices
GSD	=	Geometric standard deviation
GST	=	Glutathione-S-transferase

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H&E	=	Hematoxylin and eosin
HBBS	=	Hank's balanced buffered salt
HC	=	Hydrocarbon
HEPA	=	High-efficiency particulate air
HNO_2	=	Nitrous acid
HNO_3	=	Nitric acid
hp	=	Horse power
HP	=	Hewlett-Packard
HPB	=	4-Hydroxy-1-(3-pyridyl)-1-butanone
HPLC	=	High-performance liquid chromatography
IANR	=	Individual Animal Necropsy Record
IARC	=	International Agency for Research on Cancer
ICP	=	Inductively coupled plasma
IND	=	Investigational New Drug
ISO	=	International Standards Organization
KSU	=	Kansas State University
LAIR	=	Letterman Army Institute of Research
LC	=	Liquid chromatography
LSL	=	Life Sciences Laboratory
MCH	=	Mean corpuscular hemoglobin
MCHC	=	Mean corpuscular hemoglobin concentration
MCV	=	Mean corpuscular volume
MHV	=	Mouse hepatitis virus
MMAD	=	Mass median aerodynamic diameter
MPE	=	Method performance evaluation
MS	=	Mass spectrometry
MSD	=	Mass selective detector
MVM	=	Minute virus of mice
Na_2CO_3	=	Sodium carbonate
NCI	=	National Cancer Institute
NDA	=	New Drug Approval
NIH	=	National Institutes of Health
NNK	=	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	=	N'-Nitrosonornicotine
NO	=	Nitric oxide
NO_2	=	Nitrogen dioxide
NO_x	=	Nitrogen oxides
NPYR	=	N-Nitorosopyrrolidine
NTP	=	National Toxicology Program
OECD	=	Organization for Economic Cooperation and Development
ORR	=	Operational Readiness Review
OSU	=	Oregon State University

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PAC	=	Polynuclear aromatic compound
PAH	=	Polycyclic aromatic hydrocarbon
PAI	=	Pathology Associates, Inc.
PCB	=	Polychlorinated biphenyls
PCNA	=	Proliferating cell nuclear antigen
PI	=	Principal Investigator
PM	=	Particulate matter
PNL	=	Pacific Northwest Laboratory
PTFE	=	Polytetrafluoroethylene
PVM	=	Pneumonia virus of mice
QA	=	Quality Assurance
QAU	=	Quality Assurance Unit
RAM	=	Real-time aerosol monitors
RASS	=	Room-aged sidestream cigarette smoke
RBC	=	Red blood cells
RH	=	Relative humidity
rpm	=	Revolutions per minute
RSD	=	Relative standard deviation
RT	=	Retention time
SD	=	Standard deviation
SMVCE	=	Sperm morphology and vaginal cytology evaluation
SO_2	=	Sulfur dioxide
SOP	=	Standard operating procedure
SRM	=	Standard reference materials
TEA	=	Thermal energy analyzer
TEOM	=	Tapered element oscillating microbalance
THC	=	Total hydrocarbons
Ti	=	Titanium
TiO_2	=	Titanium dioxide
TLV	=	Threshold limit value
TPM	=	Total particulate matter
UC	=	University of California
UL	=	Underwriters' Laboratory
UV	=	Ultraviolet
VPRC	=	Volume of packed red cells
WBC	=	White blood cells
XRF	=	X-ray fluorescence
1-NP	=	1-nitropyrene
2-NF	=	2-nitrofluorene

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I. INTRODUCTION

2023685718

I. INTRODUCTION

Battelle, Pacific Northwest Laboratories (Battelle-Northwest; BNW) is pleased to submit this proposal in response to letter inquiries (October 20 and November 19, 1992) and subsequent technical discussions with Philip Morris (the Sponsor) regarding studies of the long-term inhalation effects of room-aged side-stream cigarette smoke (RASS) on the mouse respiratory tract. The RASS is used as an experimental model for environmental tobacco smoke (ETS) at concentrations of 0, 1, 3, or 10 mg total particulates per cubic meter. These exposure concentrations were carefully chosen with respect to real-life environments. In extreme conditions, the particulate concentration in a smoke-filled billiard parlor has been recorded at 0.8 mg/m^3 (Guerin et all, 1992). The high exposure concentration in this study will exceed the extreme human exposure to ETS by a factor of ~10. A diesel engine exhaust (DEE)-exposed group will be included as a positive control.

Arrangement of the technical portion of this proposal was based on our understanding of the Sponsor's desire to divide the proposal into several parts, to include:

1. A chronic, nose-only inhalation study of RASS or DEE as outlined in the sample protocol provided by the Sponsor, which includes construction and testing of the exposure and monitoring systems, and measurment of all parameters indicated in the sample protocol. Non-neoplastic and neoplastic lesions particularly in the respiratory tract will be of major interest. Animal systemic effects (e.g., changes in body and organ weights, signs of intoxication) will also be determined. Effects will be compared with those seen in the filtered-air-exposed negative and DEE-exposed positive control groups. Biomonitoring will be performed on RASS- and DEE-exposed mice.

2. A 13-week pilot study of three different strains of mice, to expose mice to room air in nose-only exposure apparatus on the same daily schedule required for the chronic study. The purpose of this study is to determine the relative ability of several strains of mice to adapt to the handling and confinement procedures required for a chronic, nose-only inhalation study.

3. A subchronic, nose-only inhalation study of RASS (two concentrations) and DEE (two concentrations). This study will utilize one strain of mice, selected by the Sponsor and BNW on the basis of the data from the pilot study (item 2 above), and other available information. This study will allow BNW to work out any technical difficulties, establish laboratory procedures, and assure the appropriate exposure concentrations selected for the carcinogenicity study.

4. Additional or substitute studies designed to examine parameters required in the Sponsor's sample protocol, and/or other studies designed to detect health effects related to inhalation of the RASS and DEE. These studies will be performed during the subchronic, nose-only study (item 3 above); selected studies will then be chosen by the Sponsor and BNW to be performed in association with the chronic study.

The general conduct of the study will comply with the Organization for Economic Cooperation and Development (OECD) Guideline 451, the National Toxicology Program (NTP) *Specifications for the Conduct of Studies to Evaluate the Toxic and Carcinogenic Potential of Chemicals in Laboratory Animals* and Good Laboratory Practices (GLP) regulations (40 CFR 792).

BNW currently has numerous contracts with NTP to conduct toxicity, carcinogenicity, and toxicokinetic studies. During the last 15 years, more than 40 chemicals have been evaluated by the inhalation route. In this project, BNW proposes to use a team of highly dedicated professional and technical staff

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whose training and experience are well suited for the proposed study. This team will be led by Dr. Earl W. Morgan, Principal Investigator, a board-certified toxicologist with more than 10 years of experience in the management of toxicity and carcinogenicity studies.

In recognition of the importance of test article generation and monitoring, an Associate Principal Investigator, Dr. Jeffrey A. Dill, is also proposed to coordinate the assignments of the exposure system, exposure generation, and chemical analysis and monitoring. Dr. Dill is an analytical chemist with 7 years of experience on NTP-type inhalation studies.

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II. TECH DISCUSSION

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II. TECHNICAL DISCUSSION

A. OBJECTIVES

Based on correspondence and discussions with the Sponsor, the following is our perception of the objectives of the proposed studies.

1. Generation and Monitoring Development Phase (Phase 1)

The objective of Phase 1 is to demonstrate that BNW is capable of developing the RASS and DEE generation and delivery systems, the animal exposure systems, the on-line aerosol and chemical monitoring and concentration control systems, and the chemical characterization systems for exposing mice to the test articles. During Phase 1, BNW will also demonstrate that the test articles will behave consistently at the desired concentrations under the specified conditions for 6 hours plus T_{90} (the time for the concentration to build to 90% of the target concentration) for 3 consecutive days. Specific tasks to accomplished during the development phase are listed below:

a. Pilot Study. During the early part of Phase 1, a pilot study using three strains of mice (C57Bl/6, B6C3F1, and CD-1) will be conducted to determine:

- Whether mice can adapt to the nose-only exposure apparatus for 6 hours/day, 5 days/week for 13 weeks
- The best strain to use for the study
- Modifications or improvements needed to the exposure system or animal handling procedure to maximize the chance of successfully conducting these studies.

b. Subchronic and Chronic Studies. During prestart efforts for the subchronic and chronic studies, BNW will:

- Develop methods of generating and monitoring RASS aerosol at 1 and 10 mg/m^3 and DEE at 3 and 10 mg/m^3
- Demonstrate the sensitivity, specificity, precision, linearity and limits of detection and quantification of the on-line aerosol and chemical concentration monitoring systems to monitor exposure units and exposure room air
- Demonstrate the sensitivity, specificity, precision, linearity, and limits of detection and quantification of the RASS and DEE chemical characterization systems
- Determine time to attain 90% of target concentration (T_{90}) following start of exposure and time to decay to 10% of the target concentration (T_{10}) following end of exposure
- Demonstrate homogeneity of concentration and particle size distribution in the nose-only exposure units
- Develop and test facility exhaust monitoring methods under actual animal exposure conditions
- Demonstrate the containment of the RASS and DEE test articles in the exposure units while simulating actual animal exposures.

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During the development phase for the generation and monitoring systems, all activities will be conducted in compliance with the U.S. Environmental Protection Agency (EPA) GLP Toxic Substances Control Act regulations 40 CFR 792. A Phase 1 Progress Report will be submitted to the Sponsor for evaluation before the start of the subchronic and chronic studies. These reports will not be delayed for detailed quality assurance (QA) auditing, however, they will present trackable data with evidence of quality control. These reports will be reviewed by BNW's QA Unit before submission of the final report.

2. Animal Study and Evaluation Phase (Phase 2)

Specific accomplishments to be achieved during Phase 2 will include conducting a 13-week subchronic study and a carcinogenicity study. The subchronic study will be conducted to evaluate effects of inhalation exposure to RASS at 1 or 10 mg/m^3 total particulate matter (TPM) and to DEE at 3 or 10 mg/m^3. During the subchronic study, some special studies proposed by BNW, as well as the parameters outlined in the Sponsor's sample carcinogenicity protocol, will be measured. The carcinogenicity study will be conducted to determine the potential for the development of neoplastic lesions during or after a life-long exposure to RASS at 1, 3, or 10 mg/m^3. Diesel engine exhaust will be used as a positive control.

During Phase 2, BNW will:

- Prepare study protocols for Sponsor approval
- Procure, receive, quarantine, randomize, and identify study animals
- Perform animal health screening
- Generate RASS and DEE at the specified concentrations during the study period with periodic calibrations of monitoring equipment
- Determine exposure unit concentration homogeneity without animals
- Determine buildup and decay times, T_{90} and T_{10}, without animals
- Determine all chemical parameters according to the frequency schedules outlined in this proposal as modified by the Sponsor
- Perform routine in-life evaluations of toxicologic parameters
- Measure respiratory minute volume
- Determine carboxyhemoglobin in blood
- Determine nicotine and nicotine metabolites in urine
- Determine particle clearance in lungs
- Determine lung particle burdens
- Evaluate hematologic parameters
- Measure organ weights
- Perform other special studies approved by the Sponsor

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- Perform necropsies and histopathologic evaluations on test animals
- Prepare and submit study reports
- Perform routine health and safety procedures, training, and monitoring
- Perform routine quality assurance inspections of Phase 2 activities and reports
- Perform routine project management functions (i.e., correspondence, monthly reports, project scheduling, and cost monitoring)
- Submit raw materials and data following the acceptance of the chronic study reports.

Toxicologic parameters to be determined in the subchronic study include:

- Mortality rate
- Body weight and body weight gain
- Clinical signs of toxicity
- Carboxyhemoglobin (COHb) in blood
- Nicotine and its metabolites in urine
- Lung burden
- Lung clearance
- Respiratory minute volume
- Hematologic evaluations
- Special studies approved by the Sponsor
- Gross lesions at necropsy
- Selected organ weights
- Microscopic evaluations of specified tissues to characterize exposure-related histopathologic changes of target organs, as well as the dose-response relationship and no-effect dose level.

The carcinogenicity study will expose mice to three concentrations of RASS, to one concentration of DEE as a positive control and to filtered air as sham-exposed controls for 24 months. Parameters to be determined for the carcinogenicity study include:

- Mortality rate
- Body weight and body weight gain
- Clinical signs of toxicity

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- Hematologic evaluations
- COHb in blood
- Nicotine and its metabolites in urine
- Lung burden
- Lung clearance
- Respiratory minute volume
- Gross lesions
- Selected organ weights
- Microscopic evaluations of specified tissues to determine and confirm exposure-related histopathologic changes of target organs as well as the dose-response relationship and no-effect dose level.

The design and conduct of this study will allow the detection of chronic toxic and neoplastic effects and assessment of the carcinogenic potential of RASS compared with DEE. Through pathologic interpretations and statistical inference (i.e., life-table methods), the following questions may be answered from this study:

- Does RASS exposure result in a significant increase in mortality? Were any increases in mortality caused by tumor formation?
- What is the time course of mortality?
- Does RASS exposure produce unique tumors that are rarely observed in control animals (type)?
- Is the rate of formation of a given tumor in a specific organ of exposed animals different from the rate in control animals or animals exposed to DEE?
- What is the multiplicity of tumors in certain tissues (sites)?
- What is the latency period leading to a particular neoplasm (time)?

These data, together with the exposure concentrations and the biologically monitored effective dose, will provide important information on the relative potency of RASS for producing tumors in mice.

B. EXCEPTIONS AND AMPLIFICATIONS

BNW costed this proposal in two phases. Phase 1 includes costs associated with those activities itemized in Section II.A.1 above. Phase 2 encompasses the total anticipated costs associated with the carcinogenicity study. The recommended subchronic study and special studies are options and are costed as such. The specific activities of Phase 2 are itemized in Section II.A.2 above.

1. Costing

The cost estimates for this proposal are based on the following assumptions:

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- Contract award by January 1994 and contract completion in December 2000
- An adequate supply of 1R4F cigarettes is to be provided by the Sponsor
- BNW's interpretation of the requirements for this study are based on information provided by the Sponsor (G.J. Patskan to B.J. Greenspan) dated 10/20/92 and 11/19/92 and a subsequent meeting with the Sponsor (G.J. Patskan and W. Reingenhaus) on 12/17/92
- The base contract includes: Pilot Study; Phase 1 activities; and a Carcinogenicity Study.
- During the pilot study, three different strains of mice will be placed in the nose-only exposure apparatus and exposed to air on the same daily schedule required for the carcinogenicity study for a period of 13 weeks. The purpose of this study is to determine the relative ability of strains of mice to adapt to the handling and confinement procedures required for a chronic, nose-only inhalation study and to provide data for the selection of an appropriate strain for the chronic study.
- During Phase 1, BNW will design, procure, construct, and test the exposure and monitoring systems for both RASS and DEE at the desirable concentration. Methods and procedures for all chemical analyses will also be developed and established.
- We will conduct a 24-month, nose-only, chronic inhalation carcinogenicity study on RASS at three exposure concentrations with a filtered-air-exposed control, and a DEE-exposed positive control. All parameters outlined in the sample protocol will be performed, with the exceptions and amplifications indicated below.
- An optional subchronic study, as well as some mechanistic studies, are proposed by BNW before the start of the carcinogenicity study. Based on data from the pilot study (described above) and other available information, one strain of mouse will be utilized. The purpose of this subchronic study is to allow BNW to resolve any technical difficulties, establish laboratory procedures, and confirm the appropriate exposure concentrations and toxicologic parameters for the chronic study. The costs of these studies are proposed separately from the base contract.

2. Schedules

At this time, BNW expects to have facilities available to perform the pilot study and the generation and monitoring development aspect of Phase 1 immediately after the proposed contract award date of January 1994. All Phase 1 activities will be completed by May 1995, followed by a 3-month approval period for the Sponsor. Phase 2 work will proceed as outlined in the Milestone Schedules, which are based on our experience with work for NTP and other industrial sponsors. The proposed contract completion date is December 2000.

3. Pilot Study to Select Mouse Strain

The C57Bl/6 mouse is the preferred strain of mice for this study because it is the only strain that has been studied and reported to develop lung tumors from exposure to both mainstream cigarette smoke and diesel engine exhaust. However, some inbred mouse strains like the C57Bl/6 are hyperactive, difficult to handle, and may not be able to tolerate the restraint tubes used for the nose-only study. Therefore, BNW proposes to perform a pilot study immediately after contract award to determine if C57Bl/6 mice can be used for this study. Two other strains, B6C3F1 and CD-1, will be included for comparison. The B6C3F1 was chosen because of the large data base available on this strain. The CD-1 is a more docile

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strain and may adapt better to the conditions of the study. Every effort will be made to train and acclimate the mice to daily handling and 6 hours of restraint in the exposure apparatus.

4. Proposed Subchronic Study

There are many uncertainties in designing and fabricating generation and monitoring systems for an inhalation study. At BNW, we have always performed some pilot and/or prechronic studies before conducting a chronic study. A prechronic study allows us to evaluate the exposure system, solve technical difficulties, establish laboratory procedures, and validate the appropriate exposure concentrations selected for the chronic study. Therefore, BNW proposes a subchronic study before the carcinogenicity study; the cost of this study is included as an optional study.

5. Diesel Engine Exhaust at 10 mg/m^3 TPM

In the past, inhalation studies conducted to determine the carcinogenic potential of DEE in mice were performed at a range between 2 and 4 mg/m^3 TPM. Heinrich et al. (1986) reported mice exposed to unfiltered DEE at 4 mg/m^3 had lower body weight compared to mice exposed to filtered DEE or control mice. Even though there is justification for a study design using positive-control DEE at the same TPM level as that in the high RASS group, BNW is concerned that 10 mg/m^3 TPM of DEE may exceed the maximum tolerated concentration for mice. Therefore, BNW recommends using two DEE exposure levels of 3 and 10 mg/m^3 in the subchronic study. After completing the subchronic study, one of these exposure levels will be chosen for the chronic study.

The TPM output of modern diesel engines is considerably less than that from older models. However, the output of nitrogen oxides has not been significantly reduced. To achieve a DEE TPM concentration of 10 mg/m^3 may require dilution of the engine exhaust by only a factor of two. This could result in a nitric oxide concentration in the exposure of as high as ~44 ppm, a value that may not be tolerated by mice on a chronic study and must be considered when choosing an appropriate DEE exposure concentration.

6. Number of Animals for Carcinogenicity Study

The OECD Guideline 451 states:

"In order for a negative test to be acceptable, it should meet the following criteria:

- No more than 10% of any group is lost due to autolysis, cannibalism, or management problems
- Survival of all groups is no less than 50% at 18 months for mice."

It is our opinion that some mice will die in the early part of the nose-only study due to inability to adapt to the exposure apparatus. BNW recommends using 60 mice/sex/group rather than 50 mice/sex/group for the study. The additional 10 mice/sex/group are intended to minimize the impact of early deaths on the validity of the study.

7. Duration of the Carcinogenicity Study

The OECD Guideline 451 states:

"It is necessary that the duration of a carcinogenicity test comprises the majority of the normal life span of the animals to be used . . . . Generally, the termination of the study

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should be at 18 months for mice. However, for certain strains of mice with greater longevity and/or low spontaneous tumor rate, termination should be at 24 months . . . ."

In this proposal, BNW recommends that the inhalation period be performed for 24 months, although the study will be terminated earlier if 75% mortality is reached in the low RASS or filter-air-exposed groups. The costs for this proposal are based on a 24-month study.

8. Clinical Observations

The protocol outline provided by the Sponsor requests "the general nonsystematic observation of all mice at least twice a day, and a careful observation according to checklist once a day on four mice/sex/group." BNW recommends that all mice be observed at least twice daily (in the early morning and late afternoon; at least 6 hours apart, 7 days/week) for mortality and moribundity. Each core study mouse will be formally examined for clinical signs of toxicity and tumor development at 4-week intervals. These observations will be recorded on a Xybion Path/Tox computer system (Xybion Medical Systems, Cedar Knolls, NJ). Signs of toxicity and/or palpable tumors first detected at times other than the formal 4-week observations will be noted and recorded on a special form designed by BNW. This information will then be entered into the computer system at the time of the next formal examination.

9. Food Consumption

Previous experience in our laboratory indicates that food consumption data generated from inhalation studies are usually not worth the effort required to obtain the data. Powdered feed, as opposed to pelleted feed, would be required for food consumption data. Malocclusion may occur and require continual clipping of the teeth. During the clipping procedure, teeth may be crushed or other physical injury may occur. Mice may urinate and defecate in the food jar, resulting in unsanitary conditions that promote infection. These incidents could result in unacceptable loss of animals and may compromise the interpretation of the study. Additionally, the individual cage unit is used to hold the restraint tube (with the animal inside) before loading on the exposure system. Placing individual food jars in each cage to measure food consumption complicates this procedure. Therefore, BNW recommends deleting the food-consumption measurements. We will make visual observations and compare food consumption of filtered-air-exposed controls and exposed groups. The cost of this proposal does not include food-consumption measurements. Should the Sponsor require measurements of food consumption, we will include this cost in our "Best and Final" negotiations.

10. Ophthalmoscopic Examinations

Eye defects were observed in about 12% of C57Bl/6 mice at Jackson Laboratories (Green, 1968). Lesions reported include micro-ophthalmia, missing eyes, and varying degrees of ophthalmia in one or both eyes. This situation progressively worsens with age and it is not unusual for more than 50% of C57Bl/6 mice to be blind at the time of terminal necropsy of a chronic study. BNW recommends deleting the ophthalmoscopic examination; therefore, the cost of this proposal does not include the ophthalmoscopic examination outlined by the Sponsor.

11. Differential White Cell Count

The protocol outline requests "light microscopic evaluation of fixed and stained blood smears to differentiate and count leukocyte types at 12 and 18 months, as well as prior to terminal necropsy." The risk of losing mice with repeated blood collection under diethyl ether narcosis (see Section II.B.12) may ultimately invalidate a study. Furthermore, a differentiated white cell count alone does not provide all the information needed to interpret the results. Therefore, BNW recommends that a complete blood count (CBC) be performed at the time of terminal sacrifice on 15 mice/sex/dose group and that leukocyte differential smears be made at this time (or at moribund sacrifice) on all mice from all dose

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groups. If statistically significant treatment-related changes are observed in the CBC, then leukocyte differential counts would be done on the remaining 45 mice/sex/dose group. A CBC includes determination of erythrocyte count, volume of packed red cells, hemoglobin concentration, red cell indices, leukocyte count, leukocyte differential, and platelet count. These analyses are determined using an automated multiparameter hematology instrument. We have costed this proposal according to this recommendation.

12. Anesthesia for Terminal Blood Collection and Euthanasia

The protocol outline provided by the Sponsor specifies that terminal blood samples be obtained while the animals are under ether anesthesia. BNW proposes that terminal blood samples be obtained from the supra-orbital sinus under 70% carbon dioxide (CO_2) anesthesia. For the past several years, our data base for hematologic parameters has been obtained using this method, which is specified in protocols for studies for NTP and other sponsors. The method we routinely use for rodent euthanasia is exsanguination following CO_2 anesthesia. If the Sponsor objects to the use of CO_2, BNW proposes using methoxyflurane as the anesthetic.

13. Disposition of Moribund Animals and Animals Unsuitable for Continued Exposure

Animals with large masses or other conditions interfering with their ability to eat, drink, urinate or defecate; major injuries or ulcers related to husbandry or treatment; debilitating conditions or other conditions indicating pain or suffering, as judged by a laboratory animal veterinarian, toxicologist, or an experienced laboratory animal specialist, will be immediately sacrificed as moribund to avoid further pain or distress. The strain of mice chosen for the study may have a relatively higher incidence of spontaneous mammary tumors that, in older animals, can attain a size that interferes with placement of the mice in the animal holding tubes of the nose-only exposure units. Because of the unique requirements of nose-only exposure, premature loss of animals to the study because of large mammary tumor masses, which would otherwise not be life-threatening must be anticipated. Therefore, BNW proposes to establish criteria with the Sponsor for determining when mammary tumor masses are too big to allow placing mice in the holding tubes. When and if this situation arises, we will continue to hold the mice in the study and sacrifice them at the time of terminal necropsy.

14. Histopathology

a. Morphometry of Laryngeal Epithelia. As described in the protocol provided by the Sponsor, we will perform morphometric measurements at several sites on the mucosal epithelium of larynges of all mice, using the hematoxylin and eosin (H&E) slides prepared for histopathologic examination. Our proposed method for performing these measurements is presented in Section III of this proposal. The cost for this procedure is included in the estimated cost of histopathology in the basic study.

b. Histology of Lungs. We will trim and prepare sections from the lungs as described in the method of Lamb and Reid (1969) for trimming rat lungs. However, since the proposed study utilizes mice, which have much smaller lungs than rats, it will be more difficult to consistently obtain single sections containing the junctions of all major branches with the mainstem bronchus of the left lung. We anticipate some variation in the number of mice per group in which sections of left lung containing all major branches of the mainstem bronchus are available.

c. Evaluation of Goblet Cell Populations in Respiratory Tract. Our proposed method for counting goblet cells in selected areas of the nose, trachea, and lungs is described in Section III of this proposal. We propose, as an alternate method to counting numbers of goblet cells per high power field as described in the method of Lamb and Reid (1969), to utilize the unit length method of Monticello et al. (1990), which expresses number of cells present per millimeter of basement membrane. This method provides a more accurate measurement of area evaluated.

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15. Carboxyhemoglobin (COHb)

The protocol outline requests that a COHb assay be conducted on three mice/sex/group using diethyl ether narcosis at 6-month intervals. BNW proposes to include 12 mice/group in the chronic study for this assay. Five mice/group will be bled at 6, 12, and 18 months under 70% CO_2 anesthesia. Five mice/sex/group from the core study animals will be used at 24 months. The mice will be allowed to recover at 6 and 12 months. Some mice may die from the procedure, others may die from natural causes by 18 months; however, it is anticipated that 12 mice will be sufficient to allow a sample group size of five mice/group at each timepoint. Five mice/sex/group will provide more statistical power.

16. Proposed Cell Proliferation Assays of Respiratory Tract Tissues in the Subchronic Study

In addition to standard histopathologic examination of respiratory tract tissues using H&E sections, the protocol provided by the Sponsor calls for morphometric examination of laryngeal epithelia at several sites, as well as examination of Alcian Blue/PAS-stained sections of respiratory tract to evaluate goblet cell populations. We propose to perform additional assays on respiratory tract tissues to evaluate epithelial cell replication rates in nose, larynx, and lung tissues of exposed and control mice using a technique already being utilized in our laboratory. Nuclei of replicating cells will be labeled immunohistochemically using a commercially available monoclonal antibody (PC 10) to proliferating cell nuclear antigen (PCNA), an endogenous nuclear protein. Use of this assay will allow us to compare cell turnover rates in mucosal epithelium of respiratory tract of exposed and control mice. A detailed description of the proposed method is provided in Section III of this proposal. The cost estimate for this optional assay is provided.

17. Proposed Protein/DNA Adducts Study in the Subchronic Study

Room-aged sidestream cigarette smoke contains many of the same compounds that are present in mainstream smoke, including polycyclic aromatic hydrocarbons (PAHs), tobacco-specific nitrosamines, 4-aminobiphenyl and other constituents (Dube and Green, 1982). The contributions of these compounds to tumor development and other health problems need to be evaluated. One method that has received considerable attention is analysis of tissues and/or body fluids for the presence of adducts to proteins and DNA. We propose to assay selected tissues from mice in the subchronic study for the presence of protein and DNA adducts. A detailed description of the proposed method is provided in Section III of this proposal. The cost estimate for this optional assay is provided.

18. Proposed Enzyme Activity Assays in the Subchronic Study

Metabolism is an important determinant of the activity of foreign compounds that enter the body (Sipes and Gandolfi, 1991). Bioactivation or deactivation, distribution, duration of activity, and excretion of a xenobiotic may all be dependent on its metabolism. Some enzymes are substrate- or functional-group-specific while others exhibit broad and overlapping substrate specificities. Metabolism is generally divided into two broad categories. Phase I metabolism involves oxidation, reduction, and hydrolysis; a prime function of these reactions is to expose functional groups (e.g., -OH, -SH, $-NH_2$, -COOH). Phase II reactions are biosynthetic reactions in which a foreign compound or a Phase I metabolite is covalently linked to an endogenous substrate resulting in a conjugate. Phase I metabolites, with their resulting functional groups, are generally electrophilic and will readily react with nucleophilic compounds in the body. These may be the Phase II substrates, in which case the conjugates formed are generally inactivated and excreted from the body, or they may covalently bind with important macromolecules in the body such as DNA and potentially lead to altered function and/or carcinogenesis (Guengerich, 1988).

We propose to look at two of the key enzymes of metabolism; the cytochrome P-450 monooxygenase system, a Phase I enzyme, and glutathione-S-transferase (GST), a Phase II enzyme. A detailed description of the proposed method is provided in Section III of this proposal. The cost estimate for this optional assay is provided.

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19. Proposed Studies to Evaluate the *In Vivo* Genotoxic Effects of RASS and Diesel Exhaust on Murine Lung Cells in the Subchronic Study

The role of chromosomal aberrations in carcinogenesis has been the subject of extensive research (Frenkel et al., 1991). Studies on some types of human cancers such as retinoblastoma (Hansen and Cavene, 1987; Knudson, 1985; Murphree and Benedict, 1984) and colorectal carcinoma (Vogelstein et al., 1988) have provided convincing evidence that chromosomal aberrations and point mutations are involved in carcinogenesis. Chromosomal aberrations produced by exposure to physical or chemical agents *in vivo* can be detected using the micronucleus test (Heddle, 1973; Heddle and Carrano, 1977). Recently we have demonstrated that micronuclei can be measured in mammalian lung cells and can be used effectively as indicators of chromosomal aberrations induced *in vivo* (Heddle et al., 1990; Khan and Heddle, 1991, 1992). A dose-dependent increase in the number of micronuclei induced in the lung cells by chemicals and x-rays was clearly evident in these experiments. Lungs are often subject to highly localized chemical concentrations during inhalation exposure. Lung tissue assays can be particularly advantageous in assessing damage to the genetic material induced *in vivo* by suspect inhaled substances. BNW has the facility and technical capability to perform the newly developed *in vivo* mammalian lung cell micronucleus test. We propose to perform this assay to determine if RASS or diesel exhaust cause chromosomal damage in the lung cells of mice during the subchronic study.

A detailed description of the proposed method is provided in Section III of this proposal. This proposed study is presented as an option and is costed as such.

20. Proposed Big Blue™ Mouse Mutation Assay in the Subchronic Study

The relationship between the mutagenicity and carcinogenicity of chemicals has been studied extensively. Most of the mutagenicity data on chemicals are derived from *in vitro* microbial and cultured mammalian cell assays. Although there is some evidence of a general correlation of mutagenicity with carcinogenicity, these *in vitro* tests are not highly accurate predictors of carcinogenicity in life-span rodent studies with many chemicals. Recently developed transgenic mouse strains provide a way to evaluate mutagenicity in specific tissues in exposed animals with a short-term assay (Kohler et al., 1990). The use of animals for mutagenicity assays is more realistic than *in vitro* tests because the functions of distribution and tissue-specific metabolism are operative. Further, cell lines used in the *in vitro* assays may have altered DNA repair mechanisms and metabolic pathways in comparison to cells in intact tissues *in vivo*.

Stratagene, Inc. (La Jolla, CA) has developed strains of mice containing the lambda bacteriophage shuttle vector (Big Blue mouse). We propose to use a Stratagene Big Blue mouse system to analyze the mutagenicity of DEE and RASS by inhalation exposure. A detailed description of the proposed method is provided in Section III of this proposal. The cost estimate for this optional assay is provided.

21. Lung Clearance and Lung Burden

The protocol outline requests that particle clearance from the lungs be measured at 15 and 18 months using a flow cytometric method to be determined. As an alternative to the use of flow cytometry, BNW proposes to chemically assess particle clearance by exposing mice to titanium dioxide (TiO_2) particles. To characterize the lung clearance curve more fully, BNW further proposes to utilize four groups of four mice/sex at 0, 3, 30, and 90 days (time points are tentative) following the exposure to TiO_2 at 15 months. The optimum time points for sampling will be determined during the subchronic study. A detailed description of the proposed method is provided in Section III of this proposal.

BNW does not have a workable method for determining lung burden for RASS at this time. Methods to determine lung burden will be developed in conjunction with the Sponsor and will be incorporated into the study design. The cost of performing the lung burden is not included in this proposal.

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III. TECH APPROACH & METHODS

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III. TECHNICAL APPROACH AND METHODS

A. STATEMENT OF WORK

The basic design for the proposed studies will comply, in general, with the study guidelines provided by the Sponsor. The studies will be performed in basic compliance with OECD guideline 451 and the NTP recommendations for carcinogenicity studies. They will also be performed in accordance with the *Guiding Principles in the Use of Animals in Toxicology* adopted by the American Society of Toxicology and will conform to GLP regulations (40 CFR 792).

B. STUDY DESIGN

The draft protocols for the proposed studies are presented in Appendix A.

1. Exposure Group Assignments

a. Pilot Study. The number of mice required for the 13-week pilot study is as follows:

Number of:	Mice		Sexes		Strains		Total
Pilot Study	15	x	2	x	3	=	90

The following is the experimental design for the pilot study:

(1) In-Life and Terminal Evaluations

Number of Mice/Strain	Daily Observations[a]	Clinical Signs[a]	Body Weights[b]	Terminal Gross Necropsy[c]
15M/15F	15M/15F	15M/15F	15M/15F	As needed

[a]All survivors to that study day (twice daily for moribundity, mortality, and clinical signs)
[b]All survivors to scheduled weighing day.
[c]Necropsy will be conducted on all mice found dead and on surviving mice showing signs of severe stress.

b. Subchronic Study. The number of mice required for this study is as follows:

Exposure Group	Number of Mice			Number of Exposure Concentrations		Total	
	Males	Females				Males	Females
Core Study–Treated	15	15	x	4	=	60	60
Core Study–Control	15	15	x	1	=	15	15
Lung Clearance–Treated	18	0	x	4	=	72	0
Lung Clearance–Control	18	0	x	1	=	18	0
Big Blue™ and Micronuclei–Treated	10	10	x	4	=	40	40
Big Blue™ and Micronuclei–Control	10	10	x	1	=	10	10
DNA Adducts–Treated	5	5	x	4	=	20	20
DNA Adducts–Control	5	5	x	1	=	5	5
Enzyme Assay–Treated	6	6	x	4	=	24	24
Enzyme Assay–Control	6	6	x	1	=	6	6
Sentinels	5	5	x	1	=	5	5
					Total =	275	185

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The following is the experimental design for the subchronic study:

(1) Basic Design

Test Group	Target Exposure Concentration (mg/m^3)	Total Number of Mice	Number of Core Study Mice[a]	Number of Special Study Mice[b]
Control	0	54M/36F	15M/15F	39M/21F
RASS	1	54M/36F	15M/15F	39M/21F
RASS	10	54M/36F	15M/15F	39M/21F
DEE	3	54M/36F	15M/15F	39M/21F
DEE	10	54M/36F	15M/15F	39M/21F
Sentinels	None	5M/5F	--	--

[a]Including five males and five females for recovery
[b]See Section III.B.1.b.(4) for allocation of these mice (p. III-3)

(2) In-Life Evaluations

Text Group	Target Exposure Concentration (mg/m^3)	Total Number of Mice	Daily Observations[a]	Clinical Signs[b]	Body Weights[b]
Control	0	54M/36F	54M/36F	15M/15F	15M/15F
RASS	1	54M/36F	54M/36F	15M/15F	15M/15F
RASS	10	54M/36F	54M/36F	15M/15F	15M/15F
DEE	3	54M/36F	54M/36F	15M/15F	15M/15F
DEE	10	54M/36F	54M/36F	15M/15F	15M/15F
Sentinels	None	5M/5F	5M/5F	--	--

[a]All survivors to that study day (twice daily for moribundity and mortality)
[b]All core study survivors to that weekly weighing day

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(3) Terminal Evaluations

Test Group	Target Exposure Concentration (mg/m^3)	Total Number of Mice	Hematology[a,b]	Gross Necropsy, Organ Weights[a]	Complete Histology, Histopathology[c]
Control	0	15M/15F	15M/15F	15M/15F	15M/15F
RASS	1	15M/15F	15M/15F	15M/15F	15M/15F
RASS	10	15M/15F	15M/15F	15M/15F	15M/15F
DEE	3	15M/15F	15M/15F	15M/15F	15M/15F
DEE	10	15M/15F	15M/15F	15M/15F	15M/15F

[a]Ten/sex/group at the time of terminal necropsy. The remaining five mice/sex/group will be evaluated after a recovery period only if abnormalities are observed in the ten mice/sex/group at terminal sacrifice.
[b]Blood from five mice/sex/group will be analyzed for COHb.
[c]All mice that die before terminal necropsy and all surviving mice in the highest exposure group with at least 60% survivors (and all survivors from higher exposure concentrations) at the time of terminal necropsy. If any lesion is encountered, which may be exposure-related, that organ will be examined in the next lower exposure group until an exposure group can be found without the exposure-related change. Target organs will only be examined in the recovery mice.

(4) Special Studies

Test Group	Target Exposure Concentration (mg/m^3)	Lung Clearance	Micronuclei and Big Blue™ Mouse Assay	DNA Adducts	Enzyme Assays
Control	0	18M	10M/10F	5M/5F	6M/6F
RASS	1	18M	10M/10F	5M/5F	6M/6F
RASS	10	18M	10M/10F	5M/5F	6M/6F
DEE	3	18M	10M/10F	5M/5F	6M/6F
DEE	10	18M	10M/10F	5M/5F	6M/6F

c. Carcinogenicity Study: The number of mice required for the carcinogenicity study is as follows:

Exposure Group	Mice		Sex		Number of Exposure Concentrations		Total
Core Study–Treated	60	x	2	x	4	=	480
Core Study–Control	60	x	2	x	1	=	120
Lung Clearance and Lung Burden–Treated	18	x	2	x	4	=	144
Lung Clearance and Lung Burden–Control	18	x	2	x	1	=	36
COHb–Treated	12	x	2	x	4	=	96
COHb–Control	12	x	2	x	1	=	24
Sentinel	15	x	2	x	1	=	30
						Total	930

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The following is the experimental design presented in tabular form for the carcinogenicity study.

(1) Basic Design

Test Group	Target Exposure Concentration (mg/m^3)	Total Number of Mice	Core Study	Lung Clearance[a]	COHb[b]
Control	0	90M/90F	60M/60F	18M/18F	12M/12F
RASS	1	90M/90F	60M/60F	18M/18F	12M/12F
RASS	3	90M/90F	60M/60F	18M/18F	12M/12F
RASS	10	90M/90F	60M/60F	18M/18F	12M/12F
DEE	–[c]	90M/90F	60M/60F	18M/18F	12M/12F
Sentinels[d]	None	15M/15F			

[a]Four mice/sex/group will be sacrificed at 0, 3, 30, and 90 days after titanium dioxide exposure. Two additional mice/sex/group are included to be used if needed.
[b]Five mice/sex/group at 6, 12, and 18 months for COHb determinations. Mice will be retained on study after the 6- and 12-month blood collections and removed from exposure after the 18-month collection. At 24 months, five mice/sex/group will be used for COHb determination.
[c]To be determined from results of subchronic study.
[d]Sentinel mice will be housed in the animal holding room and will not be exopsed

(2) In-Life Evaluations

Test Group	Target Exposure Concentration (mg/m^3)	Total Number of Mice	Daily Observations[a]	Body Weight[b]	Clinical Signs[c]
Control	0	90M/90F	90M/90F	60M/60F	60M/60F
RASS	1	90M/90F	90M/90F	60M/60F	60M/60F
RASS	3	90M/90F	90M/90F	60M/60F	60M/60F
RASS	10	90M/90F	90M/90F	60M/60F	60M/60F
DEE	–[d]	90M/90F	90M/90F	60M/60F	60M/60F
Sentinels[e]	None	15M/15F	15M/15F	– –	– –

[a]All survivors to that study day (twice daily for moribundity and mortality, at least 6 hours apart, before 10:00 am and after 2:00 pm)
[b]All core study survivors to that study day (weekly for the first 13 weeks, 4-week intervals thereafter, every 2 weeks during the last 13 weeks, if needed)
[c]All core study survivors every 4 weeks
[d]To be determined from results of subchronic study
[e]Five male and five female sentinel mice will be bled for viral serology profiles then necropsied at 6, 12, and 18 months.

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(3) Terminal Evaluations

Test Group	Target Exposure Concentrations (mg/m^3)	Total Number of Mice	Gross Necropsy and Histology	Histopathology[a]	Hematology (Complete Blood Count)[b]
Control	0	60M/60F	60M/60F	60M/60F	15M/15F
RASS	1	60M/60F	60M/60F	60M/60F	15M/15F
RASS	3	60M/60F	60M/60F	60M/60F	15M/15F
RASS	10	60M/60F	60M/60F	60M/60F	15M/15F
DEE	-[c]	60M/60F	60M/60F	60M/60F	15M/15F

[a]Complete histopathology on control, 10 mg/m^3 RASS, DEE, and early death mice. Respiratory tract and gross tumors on 1 and 3 mg/m^3, and target tissues to no-effect level.
[b]White blood count differential smear will be made on all moribund sacrificed and terminal necropsy mice, but will not be evaluated unless significant abnormalities are found on the hematology samples.
[c]To be determined from results of subchronic study.

d. Exposure Schedule. Exposure concentrations for the proposed subchronic study are 0, 1, and 10 mg/m^3 RASS and 3 and 10 mg/m^3 DEE. Exposure concentrations for the carcinogenicity study are 0, 1, 3, and 10 mg/m^3 RASS. The concentration for DEE for the carcinogenicity study will be chosen after the completion of the subchronic study.

For the chronic study, 6- to 7-week-old mice will be exposed to RASS, DEE, or to filtered air for 6 hours + T_{90} per day for up to 104 weeks. Animals will be exposed 5 days/week, excluding weekends and holidays. An exposure day missed due to equipment failure will not be made up during the study.

2. Quarantine, Housing, and Sentinel Animals

a. Quarantine Procedures. Mice will be picked up on arrival at the Pasco airport and transported directly to the Life Sciences Laboratory (LSL)-II in a BNW air-conditioned animal transport truck. Animal shipping cartons will be examined upon arrival for evidence of conditions likely to permit exposure to pathogens (soiled, wet, or otherwise damaged). If acceptable, the exterior of the cartons, except filters, will be wiped down with a chlorine dioxide solution (ABQ sterilant/disinfectant, or equivalent). The cartons will then be moved to the door of a designated quarantine room for removal of the mice in a prescribed manner to avoid direct or indirect contact of the mice with the exterior of the cartons. While being removed from the cartons, the mice will be examined for evidence of shipping stress. Acceptability at this point will depend on the condition of the animals, and the length of time the animals were in transit and environmental conditions to which they were exposed en route. After the cartons are removed, appropriate sanitation procedures will ensure cleanup of the area.

Within 48 hours of arrival, ten mice (five males and five females) from the chronic study shipment will be randomly selected for preexposure health screening. Screening procedures will include necropsy, microscopic examination of selected tissues, nasopharyngeal culture, and serologic testing for antibodies. Serologic testing will be for antibodies to Sendai virus, pneumonia virus of mice (PVM), mouse hepatitis virus (MHV), minute virus of mice (MVM), Theiler's murine encephalomyelitis virus (GDVII) and *Mycoplasma pulmonis*. Additional examination and testing will be done if health status information from the supplier is insufficient. A second health screen involving ten mice (five males and five females), randomly selected from the group remaining after mice are selected for the study, will be initiated 3 weeks after the arrival date. The 3-week period allows antibodies to develop if exposure to a pathogen occurred in transit. Procedures will be the same as those for the first health screen, except that the nasopharyngeal cultures will be omitted.

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For the Phase 1 studies, mice from each shipment will undergo health screening 3 weeks after arrival only, unless their health status has not been well defined by the supplier. If this is the case, then appropriate examination and testing will be done to screen for pathogens on arrival.

For the subchronic study, core study mice and Big Blue mice will be housed in the same room from the time of arrival. Health screening will be done only at 3 weeks after arrival. Procedures will be the same as those performed at 3 weeks for the chronic study.

The laboratory animal veterinarian will make a visual inspection of the animals to be used just prior to their release for the study. The Sponsor will be notified promptly if any significant infection or other disease condition is recognized.

b. Housing. Initially, mice will be housed approximately ten/cage in 160-in^2 wire-bottom cages. Approximately 5 days after arrival, the mice will be moved into individual wire-bottom cages with ~25 in^2 of floor space, where they will be housed for the duration of the study. Drinking water will be provided continuously by automatic watering valves, except during the nose-only exposure period. The mice will be housed in a room adjacent to the exposure room. Room temperature will be maintained at 75 ± 3°F. All animal racks will be supplied daily with clean stainless-steel excreta pans containing absorbent paper. The absorbent paper will be sterilized prior to use and will not be treated with neomycin or other bacteriostatic agents.

c. Feed. Pelleted NIH-07 open formula rodent diet will be provided. We use this feed in preference to commercial certified diets for most studies because of its wide use in government-sponsored toxicology studies (NTP) and because the NTP contaminant monitoring standards exceed those of "certified" feeds.

d. Sentinel Animals. Thirty sentinel mice will be included in the chronic study to monitor for the occurrence of infectious disease. Sentinels will be sacrificed at 6, 12, and 18 months (five males and five females at each interval). Sera will be collected from five male and five female core study mice at terminal sacrifice. Sera collected at the first three intervals will be tested at BNW for antibodies to Sendai virus, PVM, GDVII, MVM, MHV, and *M. pulmonis*. Those collected at the termination of the study will be sent to Microbiological Associates for testing for the same antibodies plus mouse adenovirus, K virus, cilia-associated respiratory bacillus, ectromelia, enzootic diarrhea of infant mice, lymphocytic choriomeningitis virus, reovirus 3, mouse cytomegalovirus and polyoma virus. Sentinel animals will also be examined for parasitic infection and selected tissues from each of the mice will be examined microscopically for evidence of infection or other disease. Should there be an indication of significant infectious disease among animals during the study, bacterial cultures or other appropriate testing will be done in order to determine the cause of the infection.

Animal health will also be monitored by routine daily observations, monthly examinations for clinical signs, and pathological examinations. The laboratory animal veterinarian will review the weight data, clinical sign data, and the necropsy reports. Any suspected disease conditions, other than those thought to be caused by experimental exposure, or as a consequence of aging, will be investigated by the veterinary staff.

Ten sentinel mice will be included in the subchronic study for viral antibody testing at the termination of the study. Testing will be for Sendai virus, PVM, GDVII, MVM, MHV, and *M. Pulmonis*.

e. Acclimatization to Exposure Units. During the quarantine period, the mice will be placed in nose-only exposure restraint tubes daily to allow them to adapt to confinement. The period of confinement will be increased gradually to 6 hours/day by the day preceding the first exposure. Only mice that adapt to the restraint tubes will be randomized, assigned to exposure concentration groups, and identified individually with a unique number.

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f. Randomization and Identification. Animals will be randomly assigned to exposure groups using body weight as a blocking variable to assure that there are no statistically significant differences in initial group mean body weight using the Xybion Path/Tox system. Animals will be identified individually by tail tattoo.

g. Nose-Only Exposure Unit. The inhalation exposures will be performed with modified Cannon flow-past nose-only exposure units (Cannon et al., 1983), developed and patented by BNW. The system is designed to provide uniform concentrations of test material at all exposure ports. The exposure system is described in Section IV.

3. Toxicology

a. Observations. Individual animal body weights for exposure and control group animals will be recorded weekly during the pilot and subchronic studies. For the chronic study, weights will be recorded weekly for the first 13 weeks and at 4-week intervals thereafter. The final weighing will occur before the scheduled terminal necropsy. If life-threatening tumors develop, or if a significant number of deaths occur in a group, the weighing frequency may be increased to every 2 weeks on approval by the Sponsor. It is anticipated that animals will be weighed every 2 weeks for the final 13 weeks of the chronic study.

All animals will be observed at least twice daily (in the early morning and late afternoon, at least 6 hours apart, 7 days/week) for mortality and moribundity. Each animal, with the exception of the sentinels, will be formally examined for clinical signs of toxicity at 4-week intervals. These observations will be recorded on the Xybion Path/Tox system. Signs of toxicity first detected at times other than at the formal 4-week observations will be noted and recorded on the Daily Observation Record.

Animals determined by a laboratory animal veterinarian, toxicologist, or toxicology specialist to be moribund or to be experiencing substantial pain or distress will be promptly euthanized. Criteria for euthanasia have been described in a standard operating procedure (SOP) to be used with this study.

If the cumulative mortality of any exposure concentration jeopardizes the ability to draw the appropriate conclusions on the carcinogenicity of the test article, the group will be terminated before the scheduled sacrifice date following approval by the Sponsor.

b. Data Collection and Review. Toxicology data will be collected by the Xybion Path/Tox system, or manually entered on specific forms. The type of data and the method of collection dictates how the data are reviewed.

An example of this process involves collecting body weights and clinical signs in both subchronic and chronic studies, as detailed below. An animal resources technician or toxicology specialist places an animal on the weighing balance, which automatically transmits the weight data to the Xybion Path/Tox system. Clinical signs are entered into the Xybion Path/Tox system using the keyboard. A copy of the data is printed when the session is completed. The toxicology specialist assigned to this study reviews these data promptly, paying particular attention to weight loss, any abnormal weight gain, or unusual clinical signs. Weights that fall outside a predetermined range are investigated as soon as possible by the toxicology specialist or animal resources technician, who in turn verifies that food and/or water were available, etc. This is documented and is stored as raw data in the study file. Printouts and review comments are routed among the scientific staff for review, including the toxicologist, who checks for accuracy and completeness, and the principal investigator/study director and clinical veterinarian, who examine the data for exposure and husbandry-related effects. Any abnormalities found during this review are checked by one of the above staff members. A weekly review involving all toxicologists, toxicology specialists, and veterinary staff is conducted and any concerns are discussed. Monthly and Study Report data are derived from this information.

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c. Off-Hour Toxicology Supervision. Weekday operations currently extend from 6:00 am to 6:00 pm. Weekend and holiday operations currently extend from 7:00 am to 3:30 pm. In order to assure complete compliance with study designs and SOPs, the toxicology staff provides off-hour supervision. A power operator monitors the building systems at all times. A staff veterinarian is on duty or on call 24 hours/day, 7 days/week.

4. Respiratory Minute Volume

Pulmonary function measurements will be made on six/mice/group during the proposed lung clearance studies in the subchronic and chronic studies and on six mice/sex/group of the core study during months 6, 12, 18, and 24 of the chronic study.

Pulmonary function will be measured by whole-body plethysmography utilizing Flow-Derived Parameters software (Buxco Electronics, Inc; Troy, NY). Animals will be placed in whole-body plethysmograph tubes similar to the containment tubes used during routine exposure. However, all ventilation holes in the tube will be sealed, a soft rubber seal will be incorporated around the animal's neck to seal the thorax from the head, and the rear animal-holding device will be replaced with an O-ring-sealed "plunger" during measurement. Air displaced by expansion-contraction of the thorax during breathing will be conducted through a 0000 Fliesch pneumotachometer.

Pressure drop across the pneumotachometer will be measured with a Validyne DP-45 pressure transducer connected to a Validyne CD-18 carrier demodulator (Validyne; Northridge, CA). The pressure signal from the demodulator will be digitally integrated and calibrated by the Buxco software to derive the tidal volume and the respiration period for each breath.

The Buxco software will record the average tidal volume, and the average respiration period for all breaths measured during each 10-second period over a 10-minute recording session beginning 10 minutes after start of exposure. The recorded data will be analyzed using Microsoft Excel spreadsheet software (Microsoft Corp., Redmond, WA) running on an Apple Macintosh (Apple Computer, Inc.; Cupertino, CA) computer. Minute volumes will be calculated as the average tidal volume multiplied by the reciprocal of the average respiration period over the 10-minute recording period. It is recognized that if the animal moves significantly within the plethysmograph, the seal may be disrupted or erroneous tidal volumes may be recorded. Therefore, all 10-second recorded average minute volumes greater than 1.5 standard deviations (SDs) from the calculated 10-minute mean minute volume will be rejected, and the mean recalculated excluding the rejected data.

5. Nicotine Metabolites in Urine

a. Introduction. Nicotine and nicotine metabolites provide specific markers of exposure to tobacco smoke. For the subchronic and chronic studies, the concentrations of nicotine in the RASS atmospheres will be determined. Determination of the amounts of nicotine metabolites excreted in the urine of the exposed animals will serve to positively demonstrate exposure and the relationship of dose to RASS exposure concentration.

BNW plans to analyze the urine for nicotine, cotinine, 3-hydroxycotinine, and the glucuronic acid conjugates of these compounds. The analytical methodology will closely follow that of Rustemeier et al. (1993). This method involves clarifying the urine, then derivatizing with diethylthiobarbituric acid (DETBA). The analytes are quantified using high-performance liquid chromatography (HPLC) with an ultraviolet (UV)-visible detector at a wavelength of 529 nm.

BNW has not been able to locate literature references citing the metabolic composition of nicotine metabolites in the urine of mice. However, nicotine, cotinine, 3-hydroxycotinine, and their glucuronic acid conjugates provide the bulk of the parent or metabolites excreted in the urine of rats and are expected to

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be similarly present in mice. Early in the method development phase for analyzing nicotine metabolites in urine, BNW plans to treat a mouse with nicotine by intraperitoneal injection. The urine will be collected and assayed for nicotine metabolites to verify the identification of major nicotine metabolites in the mouse.

After the suitability of the analyte selection has been verified, BNW will expose a limited number of animals to 1 and 10 μg/L RASS. Urine samples will be collected and analyzed to obtain an estimate of the concentration ranges for the metabolites. Thus, concentration ranges will be verified during the subchronic study by collecting urine from a limited number of animals after 4 weeks of exposure.

b. Sampling. Immediately after exposure, animals selected for urine metabolites will be placed in individual rodent metabolism cages. Urine will be collected in ice-cooled tubes for 16 hours. During urine collection, water will be provided to the animals *ad libitum*.

Urine samples will be collected at 6-month intervals during the chronic study corresponding to 6, 12, 18, and 24 months of exposure. Samples from six animals/sex/exposure group/time point will be collected from animals from the 1-, 3-, and 10-μg/L RASS exposures, as well as from the filtered-air control. After collection, urinary creatinine concentrations will be determined for each sample by an automated Jaffe reaction method using a Roche COBAS FARA instrument. The urine samples will then be stored at -70°C until nicotine metabolite analysis.

c. Analytical Methodology

(1) Sample Preparation for Free Metabolites. Urine samples (500 μl) will be centrifuged and the supernatants further clarified through ultrafiltration. An internal standard solution will be added to an aliquot of this clarified urine. The internal standard will be N'-ethylnorcotinine prepared in 6 M sodium acetate, which has been adjusted to pH 4.7. Solutions of potassium cyanide, chloramine-T and DETBA will be added as described by Rustemeier et al. (1993). After vigorous mixing, the solution will be centrifuged and the supernatant transferred to an autosampler vial for analysis by HPLC. The samples will be allowed to react for 13 to 14 minutes prior to analysis.

(2) Sample Preparation for Glucuronic Acid Conjugates. Urine samples (500 μl) will be saturated with potassium carbonate and washed with methylene chloride to remove non-polar metabolites. The aqueous phase will be neutralized with phosphoric acid, and equal amounts of a 0.05-M sodium acetate buffer will be added along with β-glucuronidase (~5000 units/ml final concentration). The samples will be incubated at 37.5°C for 18 hours and centrifuged. The supernatant will be derivatized as detailed above and analyzed by HPLC.

(3) HPLC Analysis Procedure. HPLC analysis will be carried out using a Hewlett-Packard (HP)-1050 liquid chromatograph. The UV-visible detector will be operated at a wavelength of 529 nm. Chromatographic separation will be performed using a 150-mm x 4.6-mm ID, 3-μm particle, Rainin C_{18} column at 40°C. BNW will employ the approximate mobile-phase compositions published by Rustemeier et al. (1993). These conditions will be used as a starting point and adjusted as appropriate to achieve the necessary separation.

The liquid chromatograph will be calibrated using gravimetrically prepared standards of nicotine, cotinine, and 3-hydroxycotinine. The amounts of these compounds in the urine in the nonconjugated form will be determined by direct analysis of the derivatives. The total amount of these compounds will be determined by analyzing urine samples that have undergone enzymatic cleavage. The quantity of the conjugated metabolites will be defined as the difference between the total and free metabolite.

(4) Suitability of Chosen Analytes and Range-Finding. After the general procedures for sample preparation and analysis have been established and the strain of mouse has been selected, BNW will treat a mouse with 1 mg/kg nicotine by intraperitoneal injection. The urine from this animal will

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be collected for 16 hours after injection. The urine will be analyzed for nicotine, cotinine, 3-hydroxycotinine, and the glucuronic acid conjugates of these compounds. The goal of this study is to demonstrate that the chosen analytes account for a major portion of the nicotine metabolites excreted in the urine of mice. BNW anticipates that the chosen metabolites will account for >50% of the nicotine dose. If the chosen metabolites do not account for a significant percentage of the metabolized nicotine, the contribution due to other nicotine metabolites will be investigated and major metabolites will be quantified as appropriate.

(5) Method Validation. After chromatographic separation has been demonstrated for the chosen metabolites using prepared standards, urine extracts from the 10-μg/L RASS test exposure group will be analyzed using an HP-1050 diode array detector. This instrument will provide an estimate of peak purity by an absorbance ratio technique. Good estimates of purity for the resolved metabolites in the urine extract will confirm chromatographic separation from interfering compounds. If poor peak purity is indicated, chromatographic conditions will be modified to provide the necessary separation.

To demonstrate complete enzymatic cleavage, BNW will obtain urine samples from the 10-μg/L RASS test exposure group. The urine samples will be divided into three portions. The sample portions will be allowed to undergo enzymatic cleavage for 14, 18, and 22 hours, respectively. Enzymatic cleavage will be judged complete if free metabolite concentration does not increase at the following time point. If total metabolite concentration is the same at all three time points, an 18-hour incubation time will be used for the study.

(6) Method Performance Evaluation (MPE). The basic MPE will consist of spiking the metabolite standards into urine blanks at six concentrations each, bracketing the expected concentration of the metabolites in the urine of animals exposed to 1 and 10 μg/ml RASS. The standards will be prepared from alternating preparations of duplicate stock metabolite solutions. The high and low spikes, as well as the blank, will be prepared in triplicate. The spikes will be prepared and analyzed for the free metabolite as previously described for the samples. Standard solutions of the metabolites will be prepared with concentrations matching those of the matrix spikes. These standards will be prepared in water and then derivatized as described for the samples and spikes.

BNW will assess figures of merit for the analytical methods, including linearity, sensitivity, accuracy, precision, recovery, and limits of detection and quantification.

d. Alternative Analytical Methodology Using Liquid Chromatography (LC)/Mass Spectrometry (MS)/MS. In 1994, BNW may acquire a triple quadrupole mass spectrometer for use by the Toxicology Department in support of inhalation studies. This instrument would be equipped with an atmospheric pressure chemical ionization (APCI) interface suitable for use with LC.

The high sensitivity and selectivity provided using the APCI-MS/MS would eliminate the need for sample derivatization and greatly reduce the amount of chromatographic separation needed for quantitative analysis of the urinary nicotine metabolites and glucuronic acid conjugates.

6. Lung Clearance

a. Introduction. The literature indicates that exposure to cigarette smoke and diesel particulate material may impair long-term clearance of dust from the lung (Cohen et al., 1979; McClellan et al., 1986). The purpose of lung-clearance studies is to determine whether or not exposure to RASS alters lung-clearance mechanisms in mice compared to control mice and mice exposed to DEE. Impairment of lung-clearance mechanisms can be evaluated by exposing mice to an insoluble, non-toxic aerosol and comparing lung clearance rates of normal mice to those of mice exposed to RASS and DEE. Lungs with impaired particle-clearance mechanisms will exhibit a slower particle-clearance rate (greater half-life) and higher concentrations of particulate material over time, compared to normal lungs.

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To perform lung-clearance studies, mice must be exposed to an aerosol that is relatively non-toxic and does not alter lung clearance mechanisms when administered in moderate amounts. In preparation for this proposal, BNW investigated the use of radiolabeled latex or polystyrene spheres. Advantages of using latex or polystyrene spheres are: 1) they are relatively inert; 2) they can be made in very uniform particle sizes; and 3) the presence of the radiolabel allows great sensitivity in measurement of the particle load in the lung. However, it is not possible to synthesize radiolabeled latex spheres using common labeling isotopes such as ^{3}H and ^{14}C because the radiolabel can be leached or exchanged from the sphere over time. It is possible to label latex spheres with radioisotopes of certain metals that decompose by x-ray or gamma-ray emission. However, inhalation studies using these materials pose unique occupational health and environmental hazards which must be mitigated through additional engineering controls, adding to the cost of the study. The cost of radiolabeled latex or polystyrene spheres is also quite high.

Titanium dioxide (TiO_2) is believed to be relatively non-toxic and has been used in a variety of lung clearance studies involving rodents (Ferin and Feldstein, 1978; Ferin and Leach, 1975; Greenspan et al., 1988). The relatively insoluble TiO_2 represents an acceptable choice of materials for lung clearance studies because it does not cause inflammation at low lung burdens (Klosterkotter and Buneman, 1961) and its pulmonary clearance is believed to be mediated primarily via phagocytosis by macrophages (Ferin, 1972), with relatively small amounts being translocated to the lymph nodes under normal conditions (Klosterkotter and Gono, 1971). Titanium dioxide in rodent lungs can also be measured at low concentrations relatively easily using modern atomic spectroscopic techniques such as inductively coupled plasma (ICP)-atomic emission spectrometry (AES). Accordingly, exposure of mice to TiO_2 was chosen for conducting lung-clearance studies.

Lung clearance of TiO_2 has been investigated extensively in rats, although few, if any, studies involving mice have been published. The work of Ferin and Feldstein (1978) indicates a long-term clearance half-life in Long-Evans rats of ~29 to 34 days. The study design proposed below is based on the assumption that similar behavior will be observed in mice. As discussed below, BNW proposes a pilot study that will provide information necessary to design a study specific to mice.

b. Study Design. The goal of lung-clearance studies is to determine the clearance half-life, clearance rate constant, deposition fraction, deposition rate, and pulmonary dose. This will be accomplished by exposing mice to 10 mg TiO_2/m^3 aerosol with a mass median aerodynamic diameter (MMAD) particle size between 1 and 3 μm and measuring the concentration of titanium (Ti) in the lung at various postexposure time points, using ICP-AES.

A preliminary study is proposed wherein analytical methods will be developed and validated, and the TiO_2 exposure system constructed and tested. Near the end of the development phase, 18 mice will be exposed to 10 mg TiO_2/m^3 for 6 hours/day for 3 to 5 days. Subsequently, lung assays for Ti will be used to estimate the lung burden, initial and terminal pulmonary clearance rates and half-lives, and dose to the lung. Pulmonary tidal volume measurements will be included during the exposures to facilitate calculation of deposition fraction and deposition rate. Information from the pilot study will be employed to select appropriate exposure duration and postexposure sampling times for the subchronic study.

For the subchronic study, 18 male mice/exposure group (90 mice including controls) from RASS and DEE exposures are assigned to the lung-clearance study. Immediately after termination of the subchronic RASS and DEE exposures, BNW proposes that these 90 mice be exposed to 10 mg TiO_2/m^3 for 6 hours/day for 5 consecutive days. Pulmonary tidal volume measurements will be made on six mice/exposure group, with measurements being made on one exposure group/day for 5 consecutive days. Following TiO_2 exposure, lung Ti concentrations will be determined by ICP-AES at 0, 1, and 3 days after exposure to determine the initial clearance rate and half-life. The terminal clearance rate and half-life are measured by collecting additional measurements 9, 30, and 90 days after exposure. Using an appropriate model, pulmonary tidal volume and exposure data can be used to calculate the pulmonary dose that can

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be compared to the pulmonary dose measured by ICP-AES 0 hours after exposure. These data can also be used to calculate deposition fraction and deposition rate.

This study design assumes that lung clearance may be biphasic, with the initial phase being due to ciliary clearance mechanisms and a final phase due to dissolution and other, slower translocation processes such as macrophage phagocytosis. However, if the initial clearance rate occurs more rapidly than 1 to 3 days, the design is still appropriate for a single, first-order clearance process, with a half-life of ~30 days. The design for the subchronic lung-clearance study is shown below.

Exposure Group	Male Mice[a]	Pulmonary Tidal Measurements[b]	Lung Clearance[c]
Control	18	6/group (Day 1)	3/group x 6 time points
1 mg/m^3 RASS	18	6/group (Day 2)	3/group x 6 time points
10 mg/m^3 RASS	18	6/group (Day 3)	3/group x 6 time points
3 mg/m^3 DEE	18	6/group (Day 4)	3/group x 6 time points
10 mg/m^3 DEE	18	6/group (Day 5)	3/group x 6 time points

[a]Male mice exposed (nose-only) to 10 mg TiO_2/m^3, 6 hours/day, for 5 consecutive days immediately following subchronic RASS and DEE study.
[b]On the TiO_2 exposure days indicated, pulmonary tidal volume measurements will be made on six male mice per group during TiO_2 exposures
[c]Lung burdens of TiO_2 determined by ICP-AES on three animals/group at 0, 1, 3, 9, 30 and 90 days after termination of TiO_2 exposures

Information acquired from the subchronic study will be used for similar studies for the chronic RASS and DEE studies, in which only a limited number of lung assays can be performed. For the chronic study, BNW proposes that 18 male and 18 female mice per exposure group (180 mice including controls) be removed from control, RASS and DEE exposures after 15 months. These mice will be exposed to 10 mg TiO_2/m^3 for 6 hours/day for 5 consecutive days. Pulmonary-function measurements will be performed on six male and six female mice from each exposure group during the TiO_2 exposures. The TiO_2 lung burden will be determined by ICP-AES in four mice/sex/exposure group (including controls) immediately following exposure, and 3, 30 and 90 days after exposure. The minute volume, initial lung burden, fractional deposition, and the retained lung burden at each postexposure time point will be determined. These data will be compared across exposure groups to determine differences in clearance rate or pulmonary-function measurements. These data will also be compared to those obtained during the subchronic study to discern differences arising from long-term exposure to RASS or DEE. The design for lung clearance assay in the chronic study is shown below.

Exposure Group	Male/Female Mice[a]	Pulmonary Tidal Measurements[b]	Lung Clearance[c]
Control	18M/18F	6/group (Day 1)	4/group x 4 time points
1 mg/m^3 RASS	18M/18F	6/group (Day 2)	4/group x 4 time points
3 mg/m^3 RASS	18M/18F	6/group (Day 3)	4/group x 4 time points
10 mg/m^3 RASS	18M/18F	6/group (Day 4)	4/group x 4 time points
DEE[d]	18M/18F	6/group (Day 5)	4/group x 4 time points

[a]Eighteen male and 18 female mice exposed (nose-only) to 10 mg TiO_2/m^3, 6 hours/day, for 5 consecutive days after 15 months of RASS and DEE exposures; includes two extra mice to be used if needed
[b]On the TiO_2 exposure days indicated, pulmonary tidal measurements will be made on six male mice and six female mice/group during TiO_2 exposures
[c]Lung burdens of TiO_2 will be determined by ICP-AES on four animals/group at 0, 3, 30, and 90 days after termination of TiO_2 exposures
[d]Concentration to be determined

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c. Experimental Methods

(1) Exposure Methods

(i) Generation of TiO_2. Powdered TiO_2 test material will be aerosolized using a Wright dust-feed mechanism (Model II; BGI, Inc.; Waltham, MA) connected to an air impact mill (Trost GEM-T; Garlock, Inc.; Newton, PA). The Wright dust-feed mechanism was chosen because it is capable of providing a constant output of dust over a long period of time. The test material is packed at a constant pressure (between 1000 and 2000 psi) into a sample cup. The cup is rotated downward at a fixed speed, controlled by a stepping motor, over a stationary scraper blade. Filtered compressed air passes through a groove at the outer edge of the scraper blade, entraining the dust as it is cut away. The air impact mill will be used to provide on-line reduction of the particle size if necessary. The Trost mill uses opposing air jets to cause particle-to-particle impaction and comminution. The flow rates required for the mill are compatible with the proposed nose-only exposure systems. The exposure concentration will be controlled by dilution air flow and the speed of the dust feed mechanism. BNW has performed numerous aerosol generation and characterization tests with this system.

(ii) Exposure Systems. The animals will be exposed to TiO_2 aerosol on Cannon-style nose-only exposure units, as described for the RASS and DEE exposures.

(iii) Characterization of the Exposures. Exposure concentrations will be monitored using real-time aerosol monitors (RAM; RAM-S, MIE Corporation, Billerica, MA). The instrument will be calibrated against chemically analyzed filter samples as described below. The particle size distribution of the aerosol will be determined with a Mercer-style cascade impactor sampling from the breathing zone of the animals. The material collected on each stage will be chemically analyzed as described below, and the resulting mass data will be analyzed and fit to a lognormal distribution to yield MMAD and geometric standard deviation (GSD).

(2) Analytical Chemistry Methods

(i) Test Chemical Procurement and TiO_2 Purity Assay. Reagent-grade TiO_2 sold by Fisher Scientific will be used for exposures. It is not anticipated that this material will require micronizing prior to use in the exposure system. However, BNW has the capability to micronize the test material if necessary to achieve an aerosol MMAD in the range from 1 to 3 μm.

Before starting the TiO_2 exposures in the subchronic study, BNW will assay the purity of the test material by assaying a known mass of TiO_2 for Ti using ICP-AES. Assay results will be used to determine the percent purity by mass of the test material. BNW will also analyze for elemental trace impurities in the test material using ICP-AES or ICP-MS. Test material identity will be established using x-ray powder diffraction analysis. These measurements will be performed only once.

(ii) Analysis of Exposure Concentrations. Concentrations of TiO_2 during exposures will be determined during exposures by collecting duplicate filter samples over the duration of the exposure. Duplicate filter samples will be collected on Teflon-laminated polypropylene filters (0.2-μm pore size) over the entire duration of the 6-hour exposure at a calibrated flow rate of ~0.5 L/minute using a critical orifice sampling device. The mass of TiO_2 on the filter will be determined gravimetrically. Exposure concentration (in milligrams/cubic meter) will be determined by dividing the mass of TiO_2 collected by the volume of air drawn through the filter.

BNW will also determine the purity of TiO_2 on the filters collected during exposure. Multi-element analysis of TiO_2 on filter samples from the exposure system will be performed by x-ray fluorescence (XRF) spectrometry.

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(iii) Analysis of Cascade Impactor Slides. The particle size distribution in the exposure atmosphere will be determined by collecting samples from the exposure system on a seven-stage, Mercer-style, cascade impactor (In-Tox Products, Albuquerque, NM). The mass of TiO_2 on each cascade impactor stage will be determined directly by XRF spectrometry. The relative mass of TiO_2 collected on each impactor stage will be analyzed by probit analysis (NEWCAS, Hill et al., 1977) to obtain the MMAD and the GSD for the aerosol. These measurements will be made once prior to the start of the subchronic toxicity study, and once during the subchronic and 2-year toxicity studies.

(3) Lung Burden Assay Methods.

(i) Tissue Sample Collection. The animals designated for the lung burden studies will be sacrificed at the appropriate times by inhalation of 70% CO_2 and exsanguinated via the brachial arteries. The lungs will be removed with the trachea and mainstem bronchi attached. Extraneous tissue will be trimmed, and the lobes severed from the mainstem bronchi, being careful to leave no lung tissue behind. Lung lobes will be placed in tared vials for weight determination and frozen at -20°C until analysis.

(ii) Lung Burden Assay by ICP-AES. Preweighed lung tissue samples (100 to 200 mg wet weight) will be weighed into platinum crucibles, oven dried and ignited in air to remove organic material. Lung tissue residue will then be fused with soda borax (1:4 sodium metaborate:sodium carbonate) and the fusion dissolved in 2.5 M sulfuric acid with 1% hydrogen peroxide. The resulting solutions will be analyzed for Ti by ICP-AES using the Ti (II) emission at 334.9 nm. Results will be reported as micrograms of TiO_2 per gram of lung tissue.

(4) Reports. One month prior to the start of the subchronic subchronic study, BNW will submit a Prestart Report that will include a discussion of method validation for exposure methods, lung-clearance assays, and pulmonary tidal measurements. This report will also discuss the results of the preliminary study, and final recommendations for study design for lung-clearance studies during the subchronic subchronic study. A protocol for the lung-clearance study during the subchronic study will also be submitted with the Prestart Report. A report of the findings of the subchronic lung-clearance study will be submitted as an appendix to the Final Report for the RASS and DEE subchronic toxicity study. Prior to the start of the chronic study, BNW will submit a protocol for lung-clearance studies. The Final Report on lung-clearance studies will be included as an appendix to the Final Report on the chronic study.

7. Pathology

All the pathologists, supervisors, prosectors, and histopathology technicians who will work on this study have extensive experience in conducting necropsies of laboratory rodents from prechronic and chronic inhalation studies (see Section VIII, Qualifications of Personnel).

a. Necropsy. A complete necropsy, described in detail in the BNW necropsy SOP, will be performed on all exposed and control animals that die or are sacrificed during the subchronic and chronic studies. During the pilot study, mice that die or exhibit signs of stress at the end of the study will be necropsied to determine target organs and effects. The following are amplifications to the specifications of these documents:

(1) Terminal Sacrifice. All remaining core study animals alive after 13 weeks in the subchronic study and 104 weeks of exposure in the chronic study will be euthanized and necropsied, except five mice/sex/group in the subchronic study, which will be allowed to recover for 13 weeks before sacrifice. Necropsies at terminal sacrifice will be performed in random order.

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(2) Method of Euthanasia. Moribund and terminal sacrifice animals will be euthanized by exposure to ~70% CO_2 in a closed container until unconscious, followed by exsanguination by cutting the brachial artery. The necropsy will be initiated within 5 minutes after the animal is sacrificed.

(3) Verification of Animal Identification. Each animal's identification will be confirmed by checking the tail tattoo and comparing with the identification number on the necropsy tissue jar, lid, and Individual Animal Necropsy Record (IANR) form before starting each necropsy. The tail with tattoo will be placed in fixative at necropsy, and the identification number will be verified again by the histotechnician at trimming.

(4) Collection of Specific Organs at Necropsy. Tissues to be fixed are listed below:

Adrenals
Brain (medulla/pons, cerebellar cortex, cerebral cortex)
Caecum
Colon
Duodenum
Esophagus
Eyes
Femur (including joint)
Gallbladder
Harderian glands
Heart/aorta
Ileum
Jejunum
Kidneys
Liver
Lungs (including trachea, larynx, tongue, pharynx)
Lymph nodes
Mammary gland/skin
Nose
Oral Cavity
Ovaries
Pancreas
Pituitary
Preputial/clitoral glands
Prostate
Rectum
Salivary glands
Sciatic nerve
Seminal vesicles/coagulation glands
Spinal cord (cervical, thoracic, lumbar)
Spleen
Sternum with bone marrow
Stomach
Testes/epididymides
Thigh muscle
Thymus
Thyroid (including parathyroids)
Tissue masses/tumors
Urinary bladder
Uterus
Zymbal's gland

(5) Verification by Pathologist of Collection of Adequate Samples of all Required Tissues at Necropsy. Tissue samples taken at necropsy will be placed in fixative in a tray divided into compartments labeled with the names of the organs. After the prosector has completed the necropsy, the pathologist will examine the tissues to verify the collection of adequate samples of all required tissues.

(6) Fixation of Lungs by Intra-Airway Instillation with Fixative. To standardize the degree of distension of pulmonary alveoli with fixative, lungs will be fixed by inserting a blunt needle into the tracheal lumen and infusing ethanol/acetic acid/formaldehyde/saline (EAFS) fixative at a pressure of 25 cm of water until the flow of fixative stops due to equalization of pressures. The trachea is then ligated to prevent outflow of fixative from the lungs. The pathologist will examine the infused lungs for gross lesions, then immerse the lungs in fixative.

(7) Fixation of Nasal Cavity Structures. The nasal mucosa has been shown by a number of studies at BNW to be a sensitive target tissue for rodents exposed to many test materials by inhalation. Thus, it is very important to fix these tissues adequately as soon as possible after sacrifice. This will be done by inserting a blunt needle into the pharyngeal duct from the nasopharyngeal opening and

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flushing EAFS fixative into the nasal cavity until fixative is observed coming out both external nares, then immersing the entire skull in fixative.

(8) Photographs of Gross Lesions Observed at Necropsy. Color 35-mm photographs will be prepared of representative gross lesions from target tissues or unusual gross lesions observed at necropsy or trimming. These will be identified and submitted to the Sponsor as 2" x 2" slides with the other pathology data.

(9) Provisions for Moribund Sacrifices and Necropsies on Weekends and Holidays. Trained animal resource technicians observe the animals twice a day on weekends and holidays, once in the morning and once in the evening. Animals found dead or determined to be moribund are removed from the study room and the necropsy technician on call is notified. Fixed tissues are reviewed by, and questions or problems arising during necropsy are discussed with, one of the four BNW pathologists, either at the time of necropsy or the following work day, depending on individual circumstances. If an animal is judged at any time to be moribund, the animal resource technician must obtain specific approval from one of the designated toxicology professionals before removing the animal from the study room.

b. Trimming and Slide Preparation. Trimming and slide preparation procedures will be performed as specified in the BNW SOP for trimming tissues. The following are amplifications of the specifications of these documents:

(1) Tissues Required for Histology and Histopathology in the Subchronic and Chronic Studies. The following tissues will be trimmed, processed, stained with H&E, and examined microscopically from all animals in the highest concentration group, the filtered-air controls, the positive controls, and all early deaths in the subchronic and chronic studies:

Accessory genital organs (prostate, seminal vesicles, coagulation glands)
Adrenals, right and left
Aorta (thoracic)
Brain (cerebral cortex, cerebellar cortex, pons/medulla oblongata)
Caecum
Clitoral glands
Colon
Duodenum
Epididymis
Eyes with optic nerve (if grossly abnormal)
Femur, including joint
Harderian glands
Heart
Ileum
Jejunum
Kidney, right and left
Larynx/laryngopharynx (step sections, 4 levels, laryngopharynx at the base of epiglottis)
Liver, gallbladder
Lungs (6-μm sections every 5 mm)
Lymph nodes (mandibular, mesenteric, bronchial and mediastinal)
Mammary glands
Musculature (thigh)
Nose/nasopharynx (4 levels; Young, 1981)
Oral cavity
Ovaries (mesovaries), right and left
Pancreas
Parathyroid glands
Peripheral nerve (sciatic)
Pituitary gland
Preputial glands
Rectum
Salivary glands (submandibular and sublingual)
Skin
Spinal cord (cervical, thoracic, and lumbar)
Spleen
Sternum with bone marrow
Stomach (forestomach and glandular stomach)
Testes, right and left
Thymus
Thyroid glands
Tongue
Trachea
Urinary bladder
Uterus
Zymbal's glands
All grossly visible tumors or lesions suspected being tumors

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The following tissues from the lower concentration groups will be trimmed, processed, stained with H&E, and examined microscopically in the subchronic and chronic studies:

Larynx/pharynx (4 sections)
Lungs (6-μm transverse sections every 5 mm)
Tracheobronchial lymph nodes
Nose/nasopharynx (4 sections)
Trachea (2 sections)
All grossly visible tumors or lesions suspected of being tumors
Target tissues to no-effect-level

Duplicate sections of lungs, trachea, and nose will be cut, stained with Alcian Blue/PAS, and evaluated for mucus/goblet cell hypertrophy/hyperplasia.

(2) Verification of Collection and Submission of Adequate Samples of Protocol-Required Tissues and Gross Lesions at Trimming, Slide Preparation, and Microscopic Examination. Several checkpoints are utilized in our histology laboratory and histopathology procedures to confirm trimming, embedding, microtoming, staining, and microscopic examination of protocol-required tissues and gross lesions from toxicology studies. At tissue trimming, the histotechnician indicates any abnormal tissues, and any missing or insufficient quantity of protocol-required tissues on the IANR for each animal. The technician also completes the "trim" portion of a BNW Histology Record form for each animal. This form, when completed, indicates the number of the block into which each tissue section was trimmed and the number of samples of each tissue trimmed. Similar information is entered for each animal by the histotechnician at embedding, microtoming, and tissue checkout. Before submitting slides to the pathologist, the histology supervisor or senior technician: 1) obtains the IANR form and verifies all gross lesions mentioned have been trimmed and microtomed; 2) does a slide block match up with slides 2, 5, and 7 (approximately 30% of the animal); 3) grossly examines slides for lifted up tissues and folds; 4) microscopically examines all slides for chatter and artifacts, especially all respiratory tract tissues, paying attention that the epithelial lining is complete and includes the lumen of the larynx, the cross-section and longitudinal section of trachea, and the turbinates; 5) microscopically examines the parathyroid, tracheobronchial lymph nodes and mediastinal lymph nodes and the gallbladder; and 6) after all are completed, signs off the Histology Record form with the number of slides, date checked out and initials. One copy of the Histology Record form for each animal is submitted with the IANR form and the slides to the pathologist. The pathologist indicates, at microscopic examination, each tissue examined using the Xybion Path/Tox histopathology data collection system.

In the proposed study, we will utilize the Xybion Path/Tox system to collect and tabulate histopathology data. Thus, the pathologist, histology laboratory personnel, and internal QA personnel can utilize the complete IANRs, the BNW Histology Record forms, and the computerized histopathology data files, as well as the raw data (wet tissue, blocks, and slides) to keep track of tissues trimmed, processed, and examined microscopically.

c. Special Considerations for Trimming Tissues

(1) Trimming and Slide Preparation of Laryngeal Sections. The epithelium covering the surface of the base of the epiglottis has been found to be one of the more sensitive areas to exposure-induced lesions in inhalation toxicity studies. Consistency in epiglottis sections is a key factor in the ability to detect subtle changes. We have developed a technique to obtain consistently high-quality transverse sections through: a) the base of the epiglottis in the area in which transition from stratified squamous epithelium to cuboidal/columnar ciliated epithelium normally takes place in rodents; b) the anterior ventral pouch just caudal to the base of the epiglottis; and (c) the area of the vocal folds (Renne et al., 1992a). This has allowed us to detect subtle changes in the normal anatomic features of these sensitive regions, such as squamous metaplasia, hyperplasia, necrosis, and inflammation.

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(2) Trimming and Slide Preparation of Nasal Sections. The olfactory and respiratory epithelia lining the nasal cavity in rodents have been shown by studies at BNW to be sensitive target tissues to many inhaled materials. Thus, preparation of consistently high-quality sections of nasal cavity structures will be a key factor in the detection and interpretation of early degenerative, metaplastic, and proliferative changes, as well as neoplastic lesions. Our experience in histologic preparation of nasal tissues will enable us to provide these critical tissue sections consistently. We will utilize the method of Young (1981) for trimming and microtoming nose sections.

(3) Histology of Lungs. We will trim and prepare sections from the lungs as described in the method of Lamb and Reid (1969) for trimming rat lungs. However, since mice have much smaller lungs than rats, it will be more difficult to consistently obtain single sections containing the junctions of all major branches with the mainstem bronchus of the left lung. We anticipate some variation in the number of mice per group in which sections of left lung contain all major branches of the mainstem bronchus.

d. Histopathology. Histopathology procedures will be performed as specified in the BNW SOP entitled *Histopathology*. The following are amplifications to the specifications of this document:

(1) Use of Xybion Path/Tox Histopathology Data Collection System. The Xybion Path/Tox computer system is used in our facility to collect and tabulate histopathology data for ongoing chronic studies. Data collection terminals for this system are located in each pathologist's office.

(2) Avoiding Spurious Trends in Histopathology Data. Apparent differences in the incidence of microscopic lesions among groups, which on reexamination (by the original pathologist or by a QA pathologist) are later determined not to actually be present, create a problem in toxicology studies involving microscopic examination of large numbers of tissues. Several techniques, described below, will be utilized to avoid this problem in the microscopic examination of tissues from the proposed studies.

- The pathologist will keep a dictionary of diagnostic terms used to describe microscopic lesions observed, and will minimize the number of diagnostic descriptors used. When a pathologist determines that a new morphologic descriptive term is necessary, and this term has some similarity to another term already in use, he or she will note the specific morphologic differences between the two terms and the reasons for introducing the new term in the narrative portion of the microscopic data for the first animal in which the new term is used. If the incidence of the two lesions creates potential confusion in interpretation of the data, the pathologist will briefly describe the differences in the study report.

- The pathologist will use descriptive adjectives only when necessary to differentiate similar lesions and will avoid using several descriptive adjectives for the same lesion unless absolutely necessary to differentiate induced from spontaneous lesions. When this is required, the pathologist will briefly point out the differences in the study report.

- After completing the initial microscopic examination of tissues from control and high-dose animals, the pathologist will examine the data for differences between groups. If differences in incidence and/or severity are apparent, the pathologist will then reevaluate the tissues containing these lesions from both groups in a "blind" fashion, i.e., informally randomized order without knowing the group of origin of any slide examined. This same reevaluation procedure will be done with apparent differences in the incidence of lesions in target organs examined microscopically from lower-dose groups. Reevaluation of all apparently exposure-induced lesions will be carried out before submitting the study report.

(3) Morphometry of Laryngeal Epithelium. We will measure and record the thickness of surface epithelium at several sites in the larynges of all basic study mice in which we do routine

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histopathology, using the Bioscan Optimas image analysis system (Bioscan, Inc.; Edmonds, WA). Specific areas to be measured will depend somewhat on results of microscopic examination of H&E-stained sections; and will be determined after microscopic examination of tissues and discussion with Sponsor personnel. Tentatively, we propose to measure the distance from the luminal surface to the basement membrane of the surface laryngeal epithelium at the following points:

- Base of epiglottis, in area overlying seromucous glands (Renne et al., 1992a); measurements at midsagittal point, and on either side halfway between midsagittal point and lateral edge of flat surface (total of three measurements per animal)
- Median arytenoids, using the most anterior section in which both arytenoid processes are present (Renne et al., 1992a); measure both right and left arytenoids, at a point halfway between ventral tip and most dorsal extension of medial surface (total of two measurements per animal)
- Ventral pouch; one measurement at the most ventral aspect
- Vocal folds; one measurement of the epithelium covering medial surface of each vocal fold, at the base of the fold (two measurements per animal).

We will compare the data from each of these points among exposed and control groups and analyze these data for statistical significance.

(4) Evaluation of Goblet Cell Populations in Respiratory Tract. We will prepare duplicate sections of nasal section II (at level of incisive papilla), a longitudinal section of trachea, and a longitudinal section of the left lung from all mice in subchronic and chronic studies, and stain these slides with Alcian Blue/PAS to evaluate goblet cell numbers in these tissues. We propose, as an alternate method to counting numbers of goblet cells per high power field as described in the method of Lamb and Reid (1969), to utilize the unit length method of Monticello et al. (1990), which expresses number of cells present as per millimeter of basement membrane. This method provides a more accurate measurement of area evaluated.

(5) Proposed Cell Proliferation Assays of Respiratory Tract Tissues. In addition to standard histopathologic examination of respiratory tract tissues using H&E-stained sections, the protocol provided by the Sponsor calls for morphometric examination of laryngeal epithelia at several sites, as well as examination of Alcian Blue/PAS-stained sections of respiratory tract to evaluate goblet cell populations. We propose to perform additional assays on respiratory tract tissues to evaluate epithelial cell replication rates in nose, larynx, and lung tissues of exposed and control mice. To accomplish this, we propose to utilize a technique already established in our laboratory (Renne et al., 1992b), in which nuclei of replicating cells are labeled immunohistochemically using a commercially available monoclonal antibody (PC 10; DAKO, Inc.; Carpinteria, CA) to PCNA, an endogenous nuclear protein.

PCNA is an auxiliary protein of DNA polymerase delta, an enzyme required for DNA replication. PCNA is expressed differentially during the cell cycle, and its rate of synthesis is correlated directly with the proliferative rate of cells (Celis et al., 1984). Good correlation has been demonstrated between labeling of cells with antibodies to PCNA and incorporation of tritiated thymidine or labeling with bromodeoxyuridine (BrDU) in a number of different normal tissues, tumors, and non-neoplastic proliferating cells (Hall and Woods, 1990). Use of an endogenous indicator such as PCNA has a distinct advantage over injection of exogenous labels because it provides the capability of measuring cell replication rates in routinely fixed tissues, and avoids potential problems related to injection of exogenous labeling compounds.

Use of this assay will allow us to compare cell turnover rates in mucosal epithelium of respiratory tract of exposed and control mice sacrificed for histopathology. We propose to cut additional sections of

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nose, larynx, and lungs at histology; and compare mucosal epithelial cell replication rates in these tissues by labeling nuclei with the PC 10 monoclonal antibody, counterstaining with hematoxylin, counting labeled nuclei in selected areas of the nose, larynx, and lungs, and comparing the labeling index of these tissues in exposed and control animals. For measuring labeling rates in the nose and larynx sections, we will utilize the unit length labeling index method of Monticello et al. (1990); for measuring labeling rates in lung, we will record the number of labeled alveolar epithelial nuclei in a total of 1000 nuclei examined.

Location of specific areas within the nose, larynx, and lung to be evaluated will depend somewhat on the results of microscopic examination of H&E-stained sections, and will be selected in discussions with the Sponsor after completing the microscopic examination of H&E-stained sections of respiratory tract.

BNW SOPs titled *Handling Slides; Cleaning, Screening, and Labeling Slides*; and *Shipping Slides* address storage of slides on site and final inventory, packing, and shipping pathology materials to the Sponsor.

(6) Photomicrographs of Representative Microscopic Lesions from Target Tissues. Color prints of photomicrographs of representative microscopic lesions induced in target tissues by exposure to the test materials will be prepared and submitted to the Sponsor as part of the Final Report.

8. Hematology

Hematologic evaluations will be conducted on 15 mice/sex/group randomly selected at terminal sacrifice for the chronic study. The animals will be lightly anesthetized with ~70% CO_2 in room air and blood samples collected into tubes containing potassium ethylenediaminetetraacetate (K-EDTA) via the supra-orbital sinus. In addition, blood smears will be made on all surviving mice for leukocyte differential evaluations, which will be evaluated if considered appropriate after evaluating the hematologic results from the 15 mice/sex/group. Hematologic parameters evaluated will include an erythrocyte count (red blood cells; RBC), hemoglobin (Hb) concentration, volume of packed red cells (VPRC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), leukocyte count (white blood cells; WBC), leukocyte differential count, platelet count, and cytologic assessment of all formed elements.

An Ortho ELT-8/ds blood analyzer will be used to perform the RBC and WBC counts, platelet counts, Hb, and VPRC determinations; and the red cell indices. For leukocyte differential counts, smears will be made from a well-mixed aliquot of blood using a blood film centrifuge and stained with Wright-Giemsa stain in a Wescor 7100 aerospray slide stainer. The percentage of the various leukocyte types will be determined in a minimum of 100 leukocytes and these percents converted to absolute values. All leukocyte counts will be corrected to delete nucleated erythrocytes, which will be reported independently. While performing the leukocyte differential count, the cytologic features of the erythrocytes, leukocytes, and platelets will be evaluated, as will the presence or absence of erythrocyte inclusions such as Howell-Jolly bodies. In the event that an erythrocyte count is low compared to the control group, an estimated leukocyte count will be made from the smear and compared to the instrument count.

9. Carboxyhemoglobin (COHb) Measurements

Since the COHb content of the blood is a reflection of CO dose, CO can serve as a marker for relating levels of constituents in the exposure vapor to actual dose to the subject. A variety of analytical methods are available for measuring COHb as well as CO.

BNW currently uses an IL 282 CO-Oximeter (Instrumentation Laboratories, Lexington, MA) to determine percentage of COHb in blood collected from rats and hamsters. Previous limited investigations have indicated accurate analyses in mouse blood for COHb can be obtained with the CO-Oximeter by using calculation coefficients available for rats. BNW will validate the CO-Oximeter measurements during the pilot

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study. Blood samples will be collected from a limited number of animals from the filtered-air control exposure group, as well as from those exposed to 1 and 10 mg/m^3 RASS, and 10 mg/m^3 DEE. These samples will be split and analyzed using both the CO-Oximeter and chemical-specific techniques. The chemical-specific analysis will utilize gas chromatography (GC)/MS for the determination of CO after liberation from the hemoglobin. If the CO-Oximeter results agree well with the chemical-specific analyses, the CO-Oximeter will be used for COHb analyses for the chronic study. If the two methods disagree, a decision on analysis methods will be based on the results of the validation study.

A potential problem with the CO-Oximeter analysis method is that sulfhemoglobin can cause an interfering response, giving rise to inaccurate results for COHb determinations. This could be a concern with the DEE studies, given the levels of sulfur present.

a. Sampling

(1) Animal Exposure. BNW will use a limited number of animals between 6 and 12 weeks of age for assay validation. After the generation systems have been constructed and validated, and the strain of mouse has been selected, animals will be exposed to the filtered-air control, 1- and 10-mg/m^3 RASS, and the 10-mg/m^3 DEE atmospheres for 6 hours for the COHb validation study.

(2) Sample Collection Techniques. Blood samples will be collected immediately after animal exposure. The animals will be lightly anesthetized with ~70% CO_2 and blood samples obtained from the supra-orbital sinus. Samples will be collected in tubes containing heparin as an anticoagulant. After thorough mixing, a portion of the sample will be removed for chemical-specific measurement. The remaining material will be available for CO-Oximeter analysis.

b. Analytical Methodology

(1) IL-282 Spectrophotometric COHb Determinations. A whole-blood sample is aspirated into the spectrophotometer, mixed with diluent, hemolyzed, and brought to a constant temperature in a detector cuvette. Light absorbance in the sample is measured at four specific wavelengths from which total hemoglobin is determined and the percent oxyhemoglobin, percent carboxyhemoglobin, percent methemoglobin and oxygen content are calculated.

(2) GC/MS for analysis of Carbon Monoxide. An aliquot of the collected blood sample will be used for chemical-specific determinations of CO. The sample will be placed in a 10-ml headspace vial and weighed. To liberate the CO, an acidic reagent will be added through the septum of the vial. In addition, an isotopically labeled CO standard will be added with a gas-tight syringe. The isotopically labeled internal standard will make it possible to account for difference in partition coefficient due to matrix variation. The internal standard will also provide the ability for volumetric, transfer, and detection variabilities.

Samples will be analyzed by GC/MS using an HP-5971 mass selective detector (MSD) operated in the selected ion monitoring mode. Headspace will be introduced to the gas chromatograph using an automated headspace sampler. A porous layer, open tubular, capillary column coated with molecular sieve material will be used for chromatographic separation. The GC/MS will be calibrated using volumetrically prepared samples of CO.

For the chemical-specific measurements, percent COHb will be calculated from the CO results from the GC/MS determinations and the total hemoglobin results from the CO-Oximeter. For total hemoglobin, the CO-Oximeter utilizes a cyanmethemoglobin method that is not species-dependent.

(3) Choice of Sample Analysis Methodology. The results for COHb from the spectrophotometric and chemical-specific techniques will be compared for samples from the RASS and DEE exposure. If there is good agreement between the two methods, the CO-Oximeter will be used for subse-

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quent determinations during the chronic study. In the case of a consistent bias between the two methods, the CO-Oximeter results will be corrected for the bias. Although unlikely, if a discrepancy is such that a simple bias correction cannot be validated, then the chemical-specific measurement method will be used. If such a discrepancy occurs for only diesel exhaust, then interference from sulfur may be the cause and the chemical-specific assay will be used for these samples.

10. Proposed Protein/DNA Adducts Study

Room-aged sidestream smoke contains many of the same compounds present in mainstream smoke, including PAHs, tobacco-specific nitrosamines, 4-aminobiphenyl, and other constituents (Dube and Green, 1982). The potential contributions to tumor development and other health problems need to be evaluated and one method that has received considerable attention is the formation of adducts to proteins and DNA. We propose to assay selected tissues from mice in the subchronic study for the presence of the adducts.

This type of work is based on the assumption that cigarette-smoke-induced tumors result from the production (during tobacco pyrolysis) of reactive intermediate compounds that covalently bind to DNA. These DNA modifications result in mutations, the initial step in tumor formation. Thus, adducted DNA is the most reliable biopolymer to analyze since it is the target molecule. While it follows that the total binding of carcinogen to DNA may be quantitatively related to the number of tumors observed, there is poor correlation when several carcinogens are compared. This will be important when samples from animals exposed to RASS are evaluated, because cigarette smoke contains numerous suspected carcinogens.

A purpose of the proposed study is to compare the relative potencies of sidestream smoke and diesel exhaust for the induction of adducts to proteins and DNA. This comparison is complicated since both materials result in exposure to a complex mixture of carcinogens. In addition, the chemical compositions of the two materials are different. Nevertheless, it will be possible to compare adduct levels for selected compounds because of advances in MS, improved derivatization procedures, and sample handling methods.

Several methods for analyzing DNA adducts have been developed. We propose to evaluate urine samples for modified nucleobases. Urine samples will be collected from exposed and control mice after several weeks of exposure, derivatized, and analyzed by established separation and mass spectrometric methods (Hecht et al., 1993; Shuker and Farmer, 1992). These analyses will determine whether the adducts are present and provide dosimetric information for a variety of DNA adduct species, provided that adequate concentrations are produced. The most likely adducts with adequate concentrations for analysis, are PAHs, aromatic amines and tobacco-specific nitrosamines; however, the method is also very sensitive for altered bases resulting from alkylation or oxidative damage.

Although formation of adducts to proteins such as hemoglobin and albumin are not involved in the carcinogenesis process, they may act as surrogate molecules for carcinogen binding, and therefore act as internal dosimeters. This approach has been used recently to obtain information for occupational exposure to ethylene oxide and for cigarette smoke by measuring tobacco-specific nitrosamines. We propose to conduct similar studies. For example, exposure of animals to the tobacco-specific nitrosamines, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) or N'-nitrosonornicotine (NNN) results in adducts to hemoglobin. Incubation of these hemoglobin samples under mildly basic conditions results in hydrolysis of the adduct and release of 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB). Similarly, acid hydrolysis of benzo[a]pyrene diolepoxide releases a tetrol that can also be derivatized and analyzed by GC/MS (Shugart, 1986). It has also been demonstrated that 4-aminobiphenyl, a component of cigarette smoke, forms adducts with hemoglobin at concentrations that provide dosimetric information upon analysis. We plan to compare RASS and DEE by analyzing for the compounds described above.

The experimental design for the proposed study will be as follows: Urine from five animals/sex/exposure group will be collected over a 24-hour period after 4 weeks of exposure. Blood will be collected

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the mice will be sacrificed, and the lungs and trachea will be collected and frozen at -80°C. Urine samples will be analyzed for DNA adduct metabolites; blood will be analyzed for hemoglobin adducts.

The determination of DNA or protein adducts as markers of exposure requires the use of very sensitive analytical techniques. Generally, the use of such biomarkers is hampered by inadequate sensitivity or specificity of the analytical instrumentation. However, the prospects are encouraging for achieving better sensitivity and specificity by the application of tandem MS in conjunction with either HPLC (HPLC/MS/MS) or GC (GC/MS/MS). In both instances, the use of reactant ion monitoring can often allow discrimination against materials that would have presented serious interferences to GC/MS or HPLC/MS. Furthermore, the application of either of the electrospray ionization or atmospheric pressure ionization interfaces for HPLC promises to transform HPLC/MS or HPLC/MS/MS into a practical, highly sensitive, analytical technique.

These advances in analytical instrumentation are just now finding widespread practical application and BNW is currently in the preliminary planning stages to implement such instrumentation in the Toxicology Department, where it would be used for analyzing adduct samples. If these plans are not completed, similar capability is available elsewhere within BNW. However, even with the application of such advanced techniques, the levels of adducts formed by exposure to RASS may be so low as to evade reliable measurement. If such is the case, the adduct studies would not be pursued beyond the subchronic study. However, BNW would carefully define the lower limit of quantitation of the measurement methods so that an upper bound could be established for the particular adduct(s) under study.

11. Proposed Enzyme Activity Assays

We propose to look at two of the key enzymes of metabolism; the cytochrome P-450 monooxygenase system, a Phase II enzyme, and GST, a Phase II enzyme. The cytochrome P-450 monooxygenase system consists of multiple isozymes and is one of the most important and ubiquitous of broad and nonspecific enzyme systems. Various xenobiotics have been shown to cause marked induction, up to 50- to 100-fold (Parkinson and Safe, 1987), in one or more of these isozymes. The PAHs, such as 3-methylcholanthrene, and phenobarbital are classic inducers of P-450 activity. Tobacco smoke contains over 4000 compounds, including numerous PAHs (Dube and Green, 1982).

The GST family of enzymes has received considerable attention in the last few years. These enzymes, which are present in most tissues, catalyze reactions between electrophiles and glutathione. A number of endogenous compounds including quinones, organic hydroperoxides, epoxides, alkenes and arachidonic acid are thought to be substrates for GST. The involvement of these enzymes in detoxification of carcinogens has received considerable attention since the carcinogens are eliminated by conjugation with glutathione and secretion in the bile. The involvement of this enzyme family in carcinogenesis is supported by other information. For example, increased levels of GST in tumor tissue may contribute to resistance to chemotherapeutic agents. Because of the involvement of GST in Phase II detoxification of carcinogens, it is important to evaluate these enzymes along with cytochrome P-450 activity measurements. These studies may determine the relative importance of metabolic activation and conjugation for carcinogens present in RASS.

Six mice/sex/group will be exposed for 4 weeks to RASS, DEE, or filtered air as part of the subchronic study. The mice will be sacrificed and the microsomal and cytosolic subcellular fractions from liver, lung, and kidney tissue will be prepared using differential centrifugation techniques (Guengerich, 1989; Van der Hoeven and Coon, 1974). Protein determinations will be made on these preparations using the technique of Lowery (Lowery et al., 1951) or the bicinchoninic acid method using the manufacturer's methods (Pierce, Rockford, IL). The microsomal fraction will be assayed for P-450 content (Matsubara et al., 1976; Omura and Sato, 1964). Two alkoxyresorufin enzyme assays will be used to assay for specific isozymes of P-450 (Burke et al., 1985; Lubert et al., 1985; Rutten et al., 1992). Ethoxyresorufin dealkylase activity appears to be mediated primarily by the IA1 and IA2 forms of cytochrome P-450 and is preferentially induced by PAHs. Pentoxyresorufin dealkylase activity appears to be mediated primarily by the IIB isozyme of

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cytochrome P-450 and is preferentially induced by phenobarbital. The dealkylation of these alkoxyresorufins results in a fluorescent product. The GST activity in the soluble fraction will be determined using 1-chloro-2,4-dinitrobenzene as the substrate. All three soluble classes of mouse GST have activity for this substrate. If RASS-induced changes in GST or P-450 activity are observed, the individual isoforms will be quantitated using Western blots and immunochemical procedures. Measurement of the increase in total P-450 or GST activity and, more specifically, the immunochemical identification of the specific isozyme involved can provide information on the potential mechanism of action of a xenobiotic.

12. Proposed Studies to Evaluate the *In Vivo* Genotoxic Effects of RASS and Diesel Exhaust on the Mouse Lung Cells

a. Proposed Micronuclei Study. Chromosome breakage and rearrangement, which may result in the formation of micronuclei, is one of the critical classes of change observed during the multistage progression of normal cells to cancer cells. Deep lung fibroblasts have been used to detect micronuclei induced by chemicals and radiation. To characterize this system, animals were exposed to radiation or chemicals (Heddle et al., 1990; Khan and Heddle, 1991, 1992) and the frequency of mutations or micronuclei was determined in lung fibroblasts. The chemical was given by injection; radiation was delivered either as external x-ray exposures or as inhaled radon and its progeny (Khan et al., in press). Studies were conducted in rats, mice, and Chinese hamsters and have validated the use of this end point and cell system for physical and chemical agents. This system is very sensitive and can detect damage from low levels of insult. For radiation exposure, dose-response relationships were linear. Damaged cells remained in the lung cell population with an effective half-life of about 30 days, which suggested a very slow tissue repair and made it possible to use the system as an integrator of chronic damage. Cells can be exposed for many days and the number of damaged cells will increase with increased exposure time. Such a system is very useful for conducting studies of chronic exposure of laboratory animals to environmental agents.

We propose to use transgenic mice for these studies so both clastogenic and mutagenic events can be measured in the same animal. We will use the same strain of Big Blue mouse (either the C57Bl/6 or the B6C3F1) selected for the subchronic and chronic study of RASS. The Big Blue mouse was selected for these studies on the basis of a rapidly developing data base indicating that these mice are very useful in determining the frequency of induced mutations, as well as their molecular nature.

Ten 6-week-old mice/sex/group will be exposed to either 1 or 10 mg/m^3 of RASS, to 3 or 10 mg/m^3 of DEE, or to filtered air for approximately 4 weeks as part of the subchronic study. The mice will be sacrificed following at least 2 consecutive days of exposure and the lungs will be removed aseptically. One half of each lung will be used for the micronucleus assay. The remainder will be used for the Big Blue mutagenesis assay.

The cell isolation procedure is a modification of Kahn and Heddle (1991). Briefly, the animals will be anesthetized with pentobarbital and the thoracic cavity will be opened aseptically. The lungs will be perfused with Hank's balanced buffered salt (HBBS) solution via the right ventricle of the heart. The lungs will then be removed and minced (<1-mm^3 pieces). After degassing under vacuum to remove trapped air, the tissues will be rinsed with HBBS to remove any remaining blood. The tissues will be digested in 0.25% trypsin in HBBS for 80 minutes at 37 ± 0.5°C, with agitation during the final 50 minutes of the digestion. The digestion will be stopped with the addition of RPMI medium and the debris will be allowed to settle. The supernatant will be decanted and centrifuged at 1200 rpm for 5 to 6 minutes. The pellet containing the cells will be resuspended in RPMI containing 13% fetal bovine serum and 50 µg/ml gentamicin (complete medium) and plated in single-chambered slides (Permanox; Nunc, Inc.; Naperville, IL).

The cultures will be incubated overnight at 37.5°C, 5% CO_2, and 95% relative humidity (RH). The medium will be discarded the following day to remove unattached cells and any remaining debris. The cultures will then be incubated for ~60 to 72 hours with complete medium containing 3.2 µg cyto-

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chalasin B/ml under the same conditions. The incubation will be stopped with the addition of a hypotonic solution of 0.075 M KCl and fixed with 95% methanol.

The slides will be stained with 0.01% acridine orange stain (Difco; Detroit, MI), which stains nuclear material, and scored according to the criteria of Heddle et al. (1990). The number of binucleated cells (those undergoing one cycle of cell division while in the culture media) will be recorded relative to the number of undivided cells. This will provide a measure of the rate of cell proliferation during the cell culture period and determine if the exposure caused changes in the cell cycle. The frequency of micronuclei in binucleated cells will be recorded to provide an index of chromosome damage in the cells undergoing cell division.

b. Proposed Big Blue™ Mouse Mutation Assay. Stratagene has developed strains of mice containing the lambda bacteriophage shuttle vector (Big Blue mouse). These vectors contain a *lacI* target gene that codes for a repressor of an indicator gene. The DNA of individual tissues is extracted and the phage sequences are packaged into active phage particles. When bacteria are infected with these phage and are spread onto a culture plate, they grow as a lawn of noninfected bacteria containing plaques (zones of clearing) consisting of bacteria lysed by a phage clone. Mutations that inactivate the *lacI* gene allow synthesis of β-galactosidase by infected bacteria that generates a blue color from a chromogenic substrate. Thus, blue plaques of lysed bacteria each contain a phage clone with a mutated *lacI* gene. The assay allows the measurement of mutation frequency by comparing the relative numbers of blue and normal plaques. Further, the phage from blue plaques can be amplified by growth and the DNA sequences of the mutants can be analyzed to provide a spectrum of mutations.

We propose using the Stratagene Big Blue mouse system to analyze the mutagenicity of DEE and RASS by inhalation exposure. Methods for analyzing the mutations will be those in the instruction manual provided by Stratagene. We plan to expose 10 Big Blue mice/sex/group to DEE or RASS for 4 weeks and compare these to a group of 10 Big Blue mice/sex nonexposed controls. We will extract and process the DNA from trachea, lung, and liver and analyze 400,000 plaques from each tissue for mutations. Stratagene's work to characterize the system showed that mutation frequencies were constant after more than 300,000 plaques. If the mutation frequency of the DEE-exposed group is higher than the controls, we will analyze them by DNA sequence analysis to characterize their mutations. Blue plaques will be grown in liquid culture, isolated, and the *lacI* gene sequenced by cycle sequencing using an Applied Biosystems, Inc. (ABI; Model 373A; Foster City, CA) automated sequencer. In this method, the gene is amplified in three overlapping segments by the polymerase chain reaction and the one containing the mutation is identified by single-strand conformational analysis. Thus, only one 300-base pair (bp) fragment will need to be sequenced. Sequencing directly from the phage DNA will use the protocol developed by ABI for their automated sequencer.

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IV. GENERATION & MONITORING

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IV. GENERATION AND MONITORING

A. TEST MATERIAL

1. Cigarettes

a. Specification. The test cigarette to be used to generate RASS is 1R4F, as specified by the Sponsor. The 1R4F is a filter-tipped reference cigarette with a TPM delivery of 16.9 mg/cigarette in sidestream smoke (RJ Reynolds, 1988).

b. Procurement. It is assumed that the test cigarettes will be provided by the Sponsor and are not included in the cost estimate of this proposal. They are also available from the Tobacco and Health Research Institute at the University of Kentucky (Lexington, KY). As delivered by this institute, the cigarettes are packed to contain 5,000 per case. BNW estimates that ~900 cigarettes/day will be required and ~634,000 cigarettes will be required to complete the prestart testing, and the subchronic and chronic exposures. A 13-week supply of cigarettes (~60,000) will be ordered each quarter. The capacity of BNW's freezer is sufficient to allow storage of 85,000 cigarettes between shipments.

c. Analysis. The composition and physical data on 1R4F cigarettes are summarized in Tables IV-1 and IV-2. It is expected that some physical and chemical analysis will also be provided by the Sponsor or the supplier of the test cigarettes. BNW does not plan any additional analytical measurements or tests before using these cigarettes for RASS generation.

TABLE IV-1. Composition of the 1R4F Reference Cigarette (Sullivan, 1984)

Tobacco Type	Percent Composition
Flue-cured	32.54
Burley	20.04
Oriental	11.09
Maryland	1.06
Reconstituted (Schweitzer)	27.17
Glycerol	2.80
Isosweet (sugar)	5.30

TABLE IV-2. Physical Data for the 1R4F Reference Cigarette (Sullivan, 1984)

Cigarette Rod Weight (gm)	Static Burn (sec/40 mm)	Length (mm)	Circumference (mm)	Paper Porosity (sec/50 cc)
1.072	552	84.0	25.0	22.0

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d. Storage and Inventory Control. Before signing a Confirmation of Receipt form, each case of cigarettes will be inspected and the cartons will be counted. Each carton of cigarettes will be given a unique identification number, which will be used to track the test material from receipt through usage.

The cigarettes will be stored in a locked, limited-access freezer at -16 ± 5°C. Freezer temperature will be automatically monitored four times a day. Cigarettes removed from -16°C storage will be preconditioned for at least 48 hours at room conditions (22 ± 2°C; 60 ± 10% RH).

e. Disposal. At the direction of the Sponsor, all unused test cigarettes will be returned or disposed of before the termination of the contract. Used test cigarettes will be disposed of by the chemistry group.

2. Diesel Fuel

a. Specification. Diesel fuel meeting the specifications listed below (either Type 1-D or Type 2-D) is recommended in the Code of Federal Regulations (40 CFR, 7-1-92, p. 795) for use in exhaust emission testing.

Item	Type 1-D	Type 2-D
Cetane Rating (%)	48 to 54	42 to 50
Distillation range:		
IBT, °F	330 to 390	340 to 400
10% point, °F	370 to 430	400 to 460
50% point, °F	410 to 480	470 to 540
90% point, °F	460 to 520	550 to 610
EP, °F	500 to 560	580 to 660
Gravity, API	40 to 44	33 to 37
Total sulfur, %	0.05 to 0.20	0.2 to 0.5
Hydrocarbon composition:		
Aromatics, %	8%[a]	27%[a]
Paraffins, napthenes, and olefins, %	92%	73%
Flashpoint, °F (minimum)	120	130
Viscosity, centistokes	1.6 to 2.0	2.0 to 3.2

[a]Minimum

b. Procurement. BNW has contacted several suppliers of Type 1-D and Type 2-D diesel fuel, and proposes to purchase a single batch of certified diesel fuel of sufficient quantity to complete all prestart testing, the subchronic study, and the chronic study. Currently, the estimated usage rate is ~13 gal/day and BNW estimates that 8,000 gallons of diesel fuel will be sufficient to complete the studies. Due to changes in the sulfur content of the diesel fuel that may be imposed by the U.S. Environmental Protection Agency (EPA), BNW cannot currently recommend the exact fuel type (1-D or 2-D) to be used for the chronic study. It is anticipated that by late 1993 or early 1994, new EPA specifications affecting sulfur content will be issued. Diesel fuel suppliers contacted by BNW are listed below.

Wondrack Distribution, Inc., Chevron U.S.A.
Tri-Cities, WA 99302

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Howell Hydrocarbons
1201 South Sheldon Rd
Channelview, TX 77530-0429

Sun Refining and Marketing Company
P. O. Box 1135
Marcus Hood, PA 19061-0835

Amoco Oil Company
P. O. Box 3147
Southfield, MI 48034

c. Analysis. Initial tests to be performed on the diesel fuel are comprised of American Society for Testing and Materials (ASTM) reference methods as specified below.

- Accelerated oxidation for insolubles
  D 2274
  Oxidation vessel
- Sulfur content
  D 4294
  Energy-dispersive x-ray fluorescence
- Hydrocarbon types in liquid petroleum products
  D 1319
  Fluorescent indicator adsorption
- Water and sediment determination
  D 1796
  Centrifugation

In addition, BNW proposes to fortify the diesel fuel with metal deactivators and oxidation inhibitors at the vendor-recommended concentrations.

Samples will be taken quarterly from the top and bottom of the storage tank and tested for oxidation stability, water and sediment, metal deactivator content and oxidation inhibitor content. Any water found in the bottom of the tank when samples are drawn will be tested for biological growth. If biological growth is found, a biocide (e.g., Biobor JF; U.S. Borax, Los Angeles, CA and/or Vancide 51; RT Vanderbilt, Norwalk, CT) will be added to retard further biological growth. In the event the concentration of particulate matter, organic matter, etc., exceeds the vendor-recommended concentrations, BNW will take the appropriate action (filtration, treatment, or replacement) to correct the problem; however, such contingencies have not been included in the cost estimate of this proposal.

d. Storage and Inventory Control. The diesel fuel will be stored in an 8000-gallon, triple-wall, fire-protected, above-ground storage tank. The tank will be Underwriters Laboratory (UL)-approved for Class I and Class II petroleum liquids, with a volume sufficient to store a single batch of fuel for both the prestart testing studies. The tank will be painted with a reflective material and sheltered from direct sunlight. The tank and fuel-delivery system will be constructed of materials that prevent the fuel from coming into contact with harmful metals, such as copper and its alloys (e.g., monel) or zinc, usually found in galvanized coatings. A tank sentinel system will also be included, which will be capable of detecting and alarming leaks of 0.1 gal/hour. In addition, this system will print daily volume and temperature reports.

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A water-cooled heat exchanger will be installed in the tank and a constant flow of water at a temperature between 5 and 15°C will be circulated through the heat exchanger to stabilize the temperature of the fuel. Internal temperature of the tank will be monitored automatically four times/day. An alarm will indicate if temperature limits are exceeded.

e. Disposal. BNW's Waste Management and Environmental Compliance group will dispose of excess material remaining at the end of the studies.

B. EXPOSURE ATMOSPHERE GENERATION AND DELIVERY AND EXPOSURE SYSTEMS

1. Nose-Only Exposure Unit

The inhalation exposures will be performed with modified Cannon flow-past nose-only exposure units (Cannon et al., 1983) developed and patented by BNW and depicted in Figure IV-1. The system, which is designed to provided uniform concentrations of test material at all exposure ports, incorporates two concentric plenums, one that supplies fresh aerosol to the breathing zone of each animal and one that serves as the exhaust to remove the expired and "unused" aerosol. Air flow through the unit is set so that the animals do not rebreathe their own expired air and the concentric plenum design ensures that they do not breathe a neighboring animal's expired air. A minimum flow of 250 ml/minute will be provided at each exposure port.

The exposure units used for this study will have 104 exposure ports; up to 90 will be used to expose animals. The remaining ports will either be used for sampling or will be plugged. During exposure, the animals will be contained in 3- x 14.5-cm, ventilated, transparent polycarbonate tubes with an adjustable plug at the rear opening (CH Technologies; Westwood, NJ). These tubes are mounted to the ports of the exposure unit.

Each exposure unit will be enclosed in a transparent polycarbonate plastic cabinet on a stainless-steel stand. The cabinet will isolate the exposure unit from the exposure room, but still allow observation of the animals. A door on one side of the cabinet will allow easy loading and unloading of the animals. The exposure unit will rotate on a turntable to allow access to all animals. The cabinet will be vented through a high-efficiency particulate air (HEPA) filter to the building exhaust system and will be operated at a slightly negative pressure with respect to the room.

The exposure units will be operated in pairs (Figure IV-2) at essentially identical concentrations; one for exposure of female mice, the other for males. Single units for each concentration will be used for the subchronic study with both males and females being exposed simultaneously in each unit. On-line monitoring of TPM and CO will be performed through nose ports in one of the pair of exposure units. This method of on-line monitoring will reduce the number of monitoring systems required, thus reducing costly maintenance and calibration. During prestart system validation, daily filter samples will demonstrate that the TPM and CO concentrations in the two units are essentially identical.

2. System Layout

Figure IV-3 shows a diagram of the rooms in the basement of LSL-II that BNW proposes to use for the DEE and RASS studies.

The diesel engine, dynamometer and dynamometer control, and data acquisition system will be contained in Room 511. This room will also contain a spare engine. Both engines will be mounted on movable stands to facilitate quick and easy exchange. A tank containing sufficient fuel for 1 day of exposure will also be housed in this room, with the main fuel storage tank located in a fenced, paved area outside the building.

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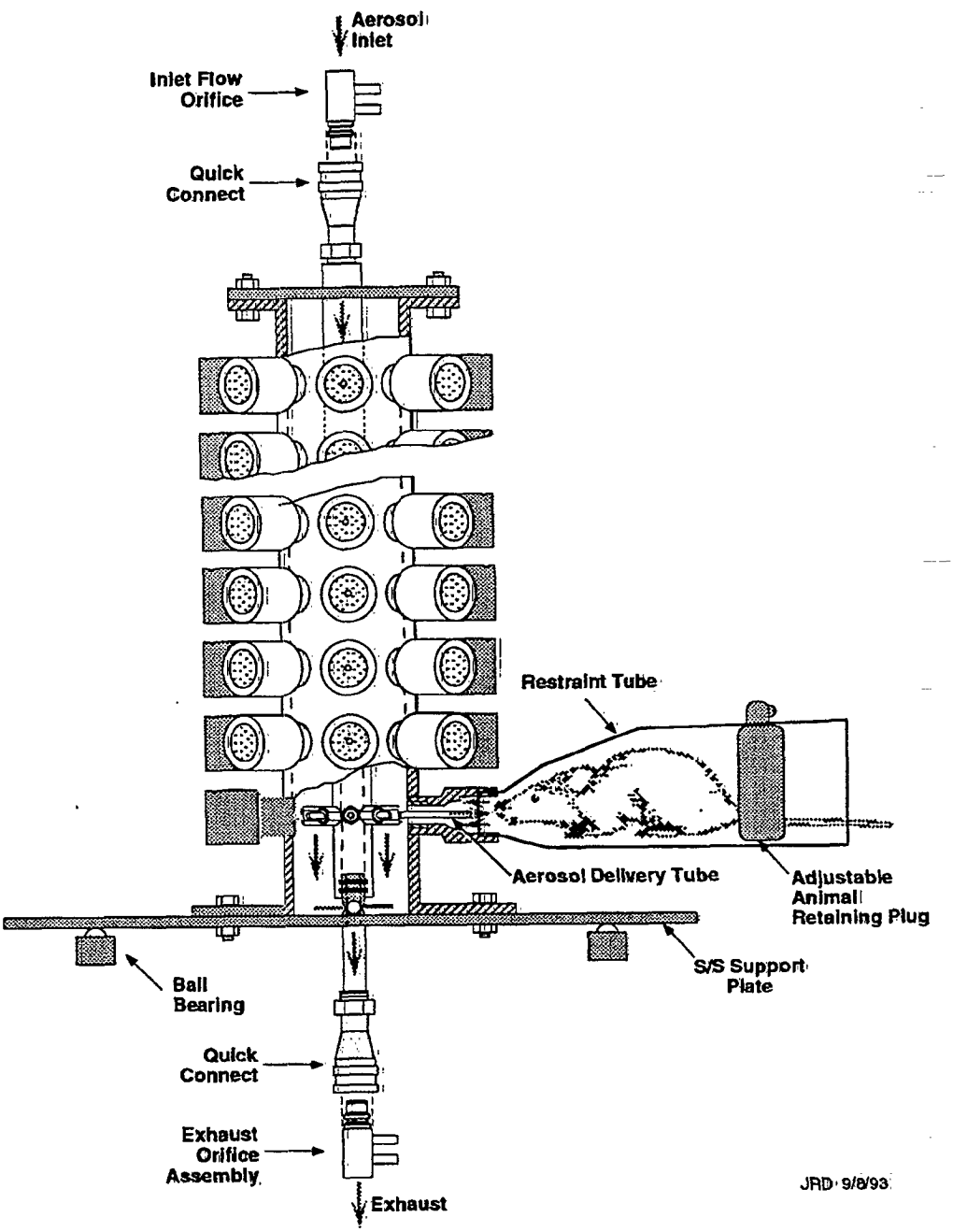

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FIGURE IV-1. Diagram of the Modified Cannon Nose-Only Exposure Unit

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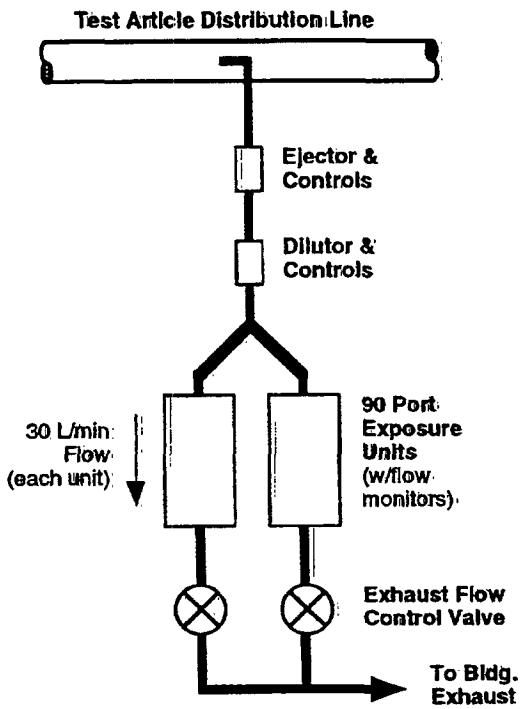

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FIGURE IV-2: Diagram of Paired Arrangement of Nose-Only Exposure Units

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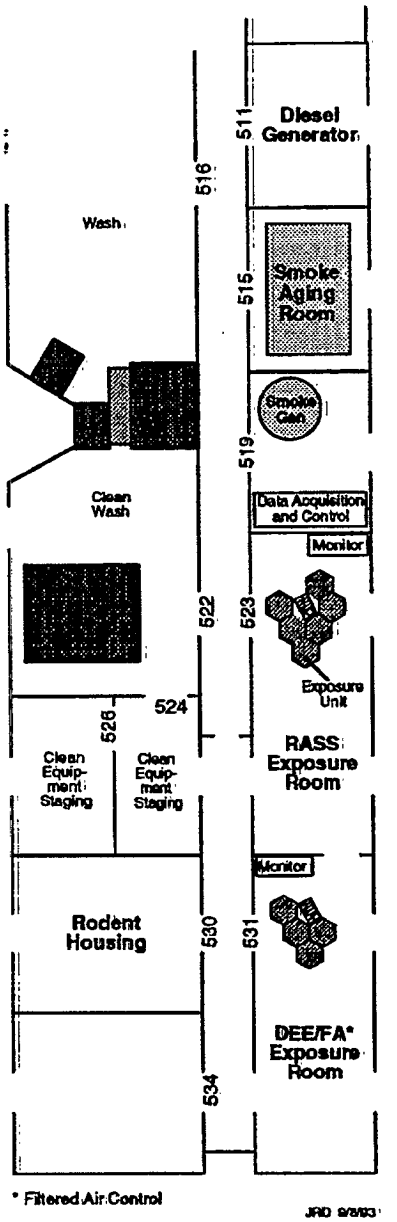

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FIGURE IV-3. Portion of the Basement of the Life Sciences Laboratory-II Building Showing Room Layout of the Room-Aged Sidestream Cigarette Smoke (RASS) Generator and Aging Room, the Diesel Engine Exhaust (DEE) Generator, the Animal Exposure Rooms, and the Animal Housing Room

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The cigarette smoke exposure machine will be located in Room 519. The smoke aging room will be located in Room 515. Room 519 will also contain the automated exposure data acquisition and control system. An exposure technician can attend to both the routine operation of the cigarette smoking machine and the data acquisition and control system.

The three RASS exposure unit pairs for the chronic study will be located in Room 523. A delivery line will carry the RASS from the aging room to the exposure units. The two DEE exposure unit pairs, as well as the two filtered-air exposure unit pairs, will be located in Room 531. A heated delivery line will carry the diesel exhaust from the engine room to a location near the exposure chambers.

All the animals will be housed in Room 530 and transported to the exposure rooms prior to exposure.

3. RASS Generation and Delivery System

a. Introduction. The cigarette smoke test article will be RASS, which is taken as an experimental model for ETS. A schematic diagram of the RASS generation system and aging room is shown in Figure IV-4. A diagram of the RASS delivery system is shown in Figure IV-5.

b. Smoking Machine. Sidestream smoke will be collected as the test cigarettes, 1R4F, are smoked by a 30-port smoking machine (Model 1300C, CH Technologies; Westwood, NJ). Standard puffing conditions (Table IV-3) following guidelines of International Standards Organization (ISO) Standards #3402, #4387 and #3308 will be used.

A stainless-steel cone will be fitted over the smoking machine to extract the sidestream smoke. Conditioned dilution air (22 ± 2°C and 60 ± 10% RH) will be mixed with the smoke before being injected into a smoke-aging room by a controlled-flow fan. Mainstream smoke from the smoking machine will be passed through a filter system to remove the majority of the particulate material before it is routed to the building exhaust.

c. Smoke-Aging Room. The sidestream smoke will be aged in a 30-m^3 (~10- x 13- x 8-ft) room contained in Room 515 of LSL-II. The aging room will be constructed of wallpaper-covered drywall attached to steel studs. The floor will be epoxy-paint-covered concrete. A sealed door will allow access to the aging room. Lighting will be provided by a single incandescent lamp, which will normally be turned off during smoke generation. Optimal lighting conditions that may affect RASS composition will be considered in cooperation with the Sponsor. The aging room will be maintained at a temperature of 22 ± 2°C with 60 ± 10% RH. A constant air flow of 60 m^3/hour through the room will satisfy the required ventilation rate of two air changes/hour and a mean aging time of ~0.5 hour. Mixing fans will be installed in the mixing room to ensure a uniform atmosphere. TPM buildup and decay times in the aging room will be evaluated experimentally during the pre-exposure testing phase.

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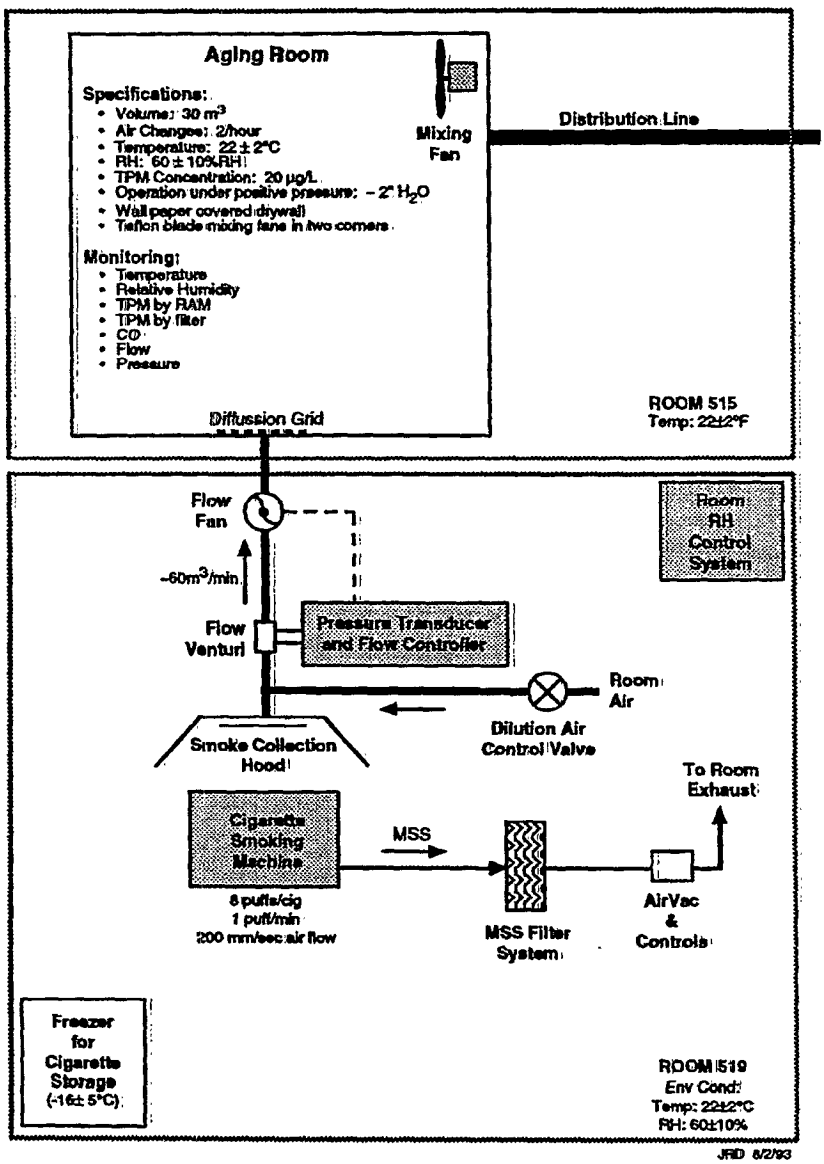

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FIGURE IV-4. Schematic Diagram of the Sidestream Cigarette Smoke Generation System and Aging Room

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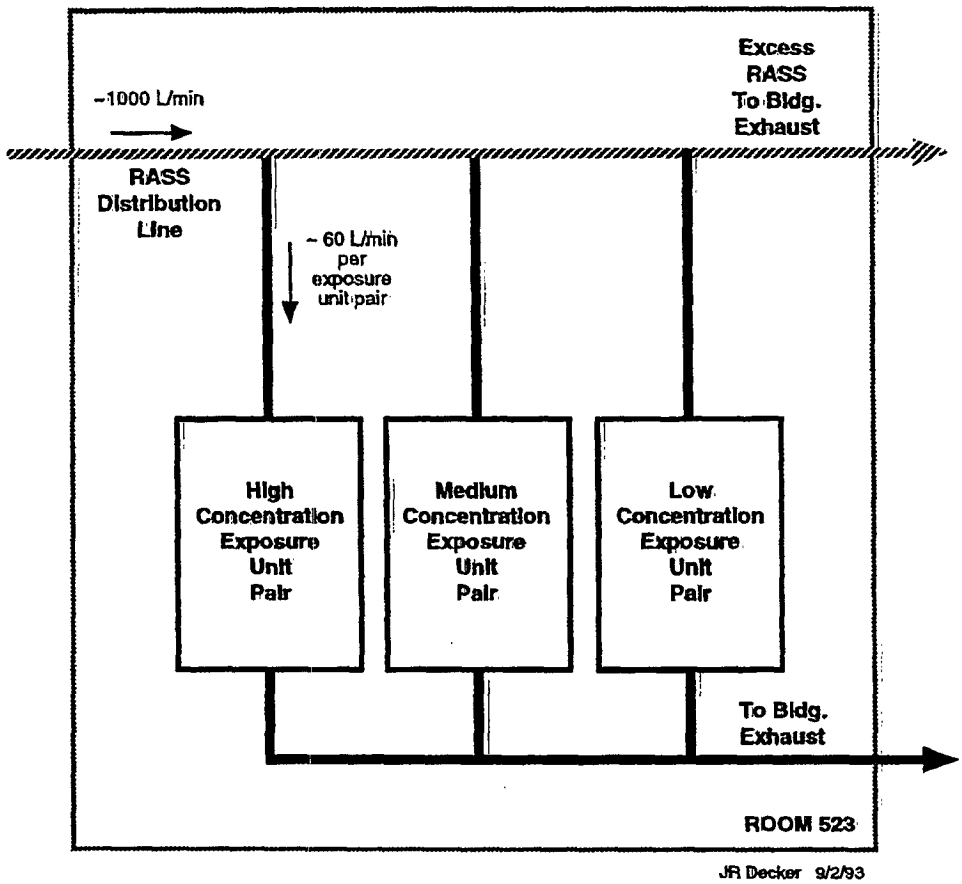

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FIGURE IV-5. Schematic Diagram of the Room-Aged Sidestream Cigarette Smoke Distribution and Exposure System

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TABLE IV-3. Conditions of Smoke Generation and Collection

Puff volume	35 ml
Puff duration	2 seconds
Puff number[a]	8
Puff frequency	One puff/minute
Air velocity around the cigarette	200 mm/second

[a]ISO Standard #4387 defines the standard butt length as the greatest of three lengths: 23 mm, length of the filter plus 8 mm, or overwrap plus 3 mm. In the smoking machine proposed, a "butt length switch" controls the length of the cigarette rod smoked, before the butt is automatically dumped into an ashtray. The switch is set at the factory for a 35-mm butt length, but can be adjusted for other lengths. An alternative is taking a set number of puffs (e.g., six puffs after the first lighting puff) from each cigarette instead of smoking them to a fixed butt length. Because the number of puffs required to reach a consistent butt length can vary from cigarette to cigarette, this alternative will ensure a consistent and whole puff number for each cigarette.

Aerosol and CO concentrations will be monitored in the aging room for 30 minutes before the start of each exposure. TPM concentration in the aging room and in the distribution line should be at least twice as high as the maximum chamber concentration desired (i.e., 20 mg/m^3). The loss of particulate material from smoke during dilution and aging has been reported between 20 and 50% (Baker and Proctor, 1990; Guerin et al., 1992; Ingebrethsen et al., 1988; Pritchard et al., 1988). An approximate estimate of the cigarette consumption rate can be made as shown below. This estimate includes a factor of 1.5 for the loss of TPM due to dilution and aging, assumes 16.9 mg TPM delivery/cigarette, and allows for 1 hour of generation, prior to exposure, for buildup and mixing/aging (i.e., a total of 7 hours of generation).

$$\frac{1.5 \times 20\ mg\ TPM/m^3 \times 60\ m^3/hour \times 7\ hours/day}{16.9\ mg\ TPM/cigarette} = 746\ cigarettes/day$$

BNW estimates that ~13 cigarettes will be smoked at the same time. Assuming that 15 cigarettes must be loaded every 7 minutes, a total of 900 cigarettes/day will be required. Since the capacity of the automatic loading device on the smoke generator is ~400 cigarettes, the reservoir will be manually loaded about every 3 hours.

Temperature and RH in the aging room will be automatically monitored approximately every 2 hours during smoke generation. Air pressure in the aging room, which will be slightly positive compared to the laboratory room, will be manually recorded daily. Aerosol and CO concentration will be automatically recorded approximately twice per hour.

d. Delivery System. The entire flow from the aging room will be delivered to the exposure rooms via a stainless-steel delivery tube. The required flow of RASS will be withdrawn from this delivery line by a pneumatically controlled ejector tube, mixed with conditioned air to achieve the proper concentration, and injected into the nose-only exposure unit (Figure IV-5). Exhaust from the exposure unit will be routed through a flow-control valve to an exhaust tube maintained at a negative differential pressure

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by a pneumatic pump. All RASS exhaust will be passed through a filter system to remove the majority of the particulate material before being routed to the building exhaust.

4. DEE Generation and Delivery System

a. Overview. A schematic diagram of the proposed DEE generation system is shown in Figure IV-6. The DEE will be generated from a light-duty pickup truck engine on a shock-mounted engine stand attached to a dynamometer system. The dynamometer will be programmed to simulate the EPA *Urban Dynamometer Driving Schedule* (40 CFR, Chapter 1, Pt. 86, Appendix I). This test sequence is normally performed on a chassis dynamometer utilizing the complete vehicle and drive train. However, BNW will simulate this using a motoring dynamometer that will control both the acceleration and deceleration of the engine. The data acquisition and control system associated with the dynamometer (Section IV.B.4.d) will be capable of simulating the proposed cycle.

A schematic diagram of the proposed DEE distribution and exposure system is shown in Figure IV-7. A constant flow of DEE from the engine will be delivered from a plenum downstream from the engine muffler to the exposure rooms through heated stainless-steel delivery lines. Flow will be maintained by a pneumatic pump located at the downstream end of the delivery tube. The required flow of DEE will be withdrawn from this delivery line by a pneumatically controlled ejector tube, mixed with conditioned air to achieve the proper concentration, and injected into the nose-only exposure unit. Exhaust from the exposure unit will be routed through a flow control valve to an exhaust tube maintained at negative relative pressure by a pneumatic pump. All DEE exhaust will be routed to the building exhaust.

The exhaust must be at room temperature on entry into the nose-only exposure units. To accommodate the required reduction in temperature, the exhaust will be cooled gradually as it traverses the duct leading from the engine to the exposure unit. If further cooling is necessary, a cooling plenum will be placed just upstream from the exposure unit.

The engine will be supplied with clean air taken from the animal facility corridor. Fuel will be supplied from a 20-gal tank, which will be kept full by a pump connected to a larger tank located outside the building.

All engine control functions will be performed by the dynamometer control system, including starting, acceleration, and deceleration. The dynamometer control system will also monitor the operating conditions of the engine. A second, identical engine will be ready on a test stand in the event that the primary engine malfunctions. Both engines will undergo break-in as specified by the manufacturer before being used in the studies.

Routine maintenance will be performed on the primary engine during nonexposure periods. This will include oil and filter changes at every 3000-mile equivalents. Other maintenance will be performed as recommended by the engine manufacturer and recorded in an operating log.

b. Simulating the EPA *Urban Dynamometer Driving Schedule*. The EPA *Urban Dynamometer Driving Schedule* is a protocol for performing emissions testing from light- and medium-duty vehicles. The procedure calls for a series of accelerations, decelerations, and stops over a period of ~23 minutes, and a 7.5-mile distance. This cycle is shown graphically in Figure IV-8. The EPA protocol specifies the vehicle speed on the chassis dynamometer. Upon installation of the engine and dynamometer, a calibration of speed versus revolutions per minute (rpm) and torque will be performed. These data will be used to set the appropriate rpm and torque corresponding to the EPA-specified protocol. Before the start of exposures each day, the engine will warm up during one complete cycle, after which it will idle until the animals are ready for exposure. The cycle will be repeated ~16 times during the 6-hour + T_{90} exposure regimen.

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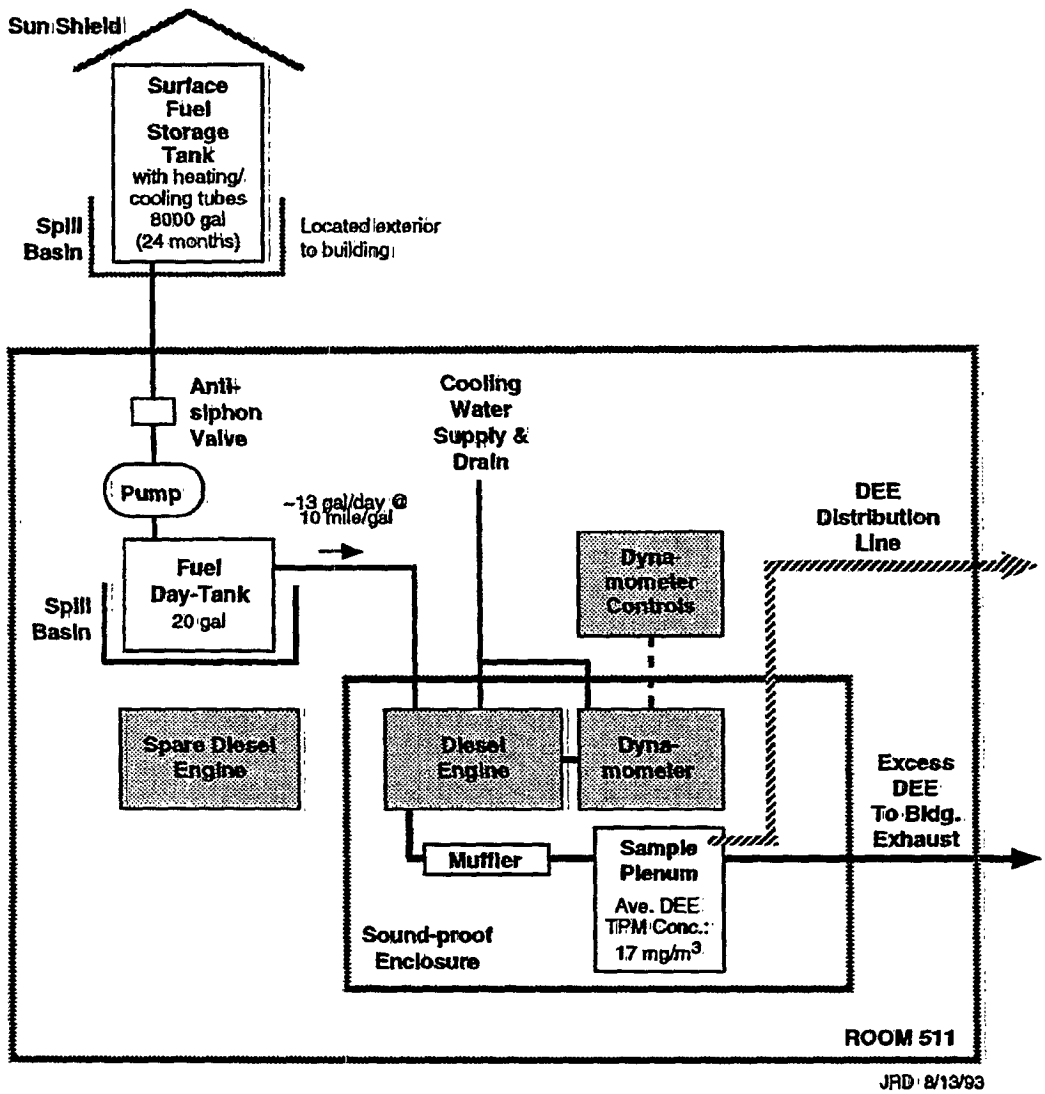

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FIGURE IV-6. Schematic Diagram of the Diesel Engine Exhaust Generation System

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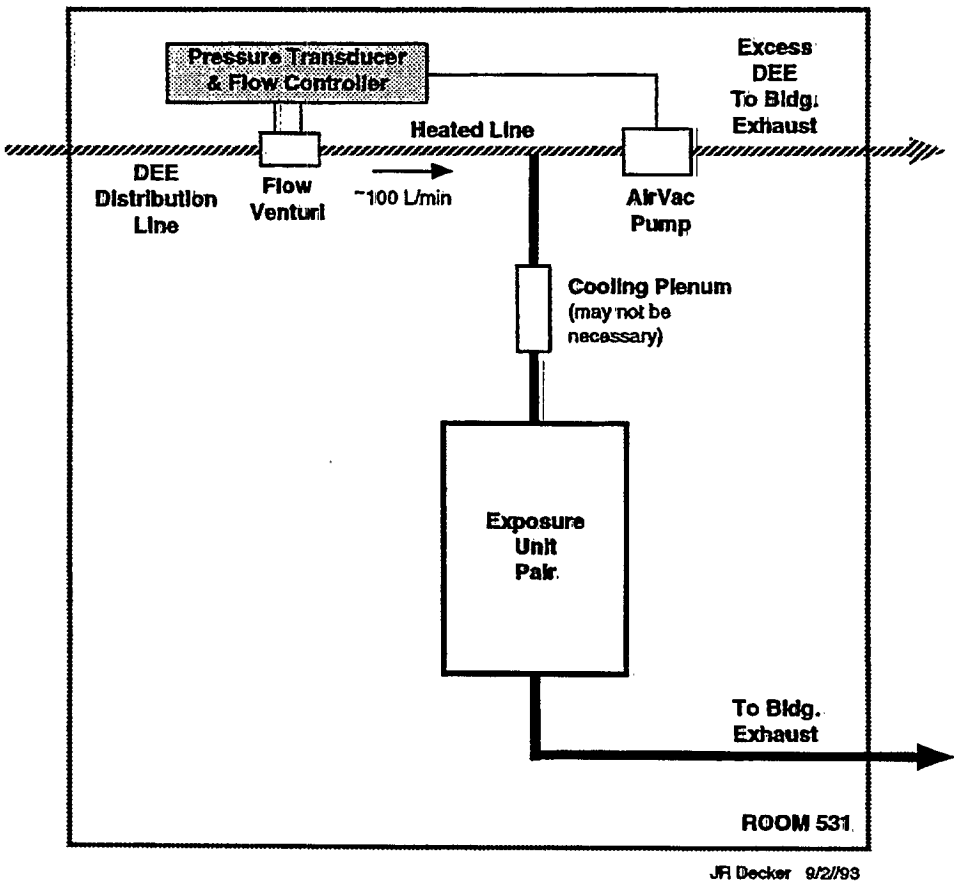

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FIGURE IV-7. Schematic Diagram of the Diesel Engine Exhaust Distribution and Exposure System

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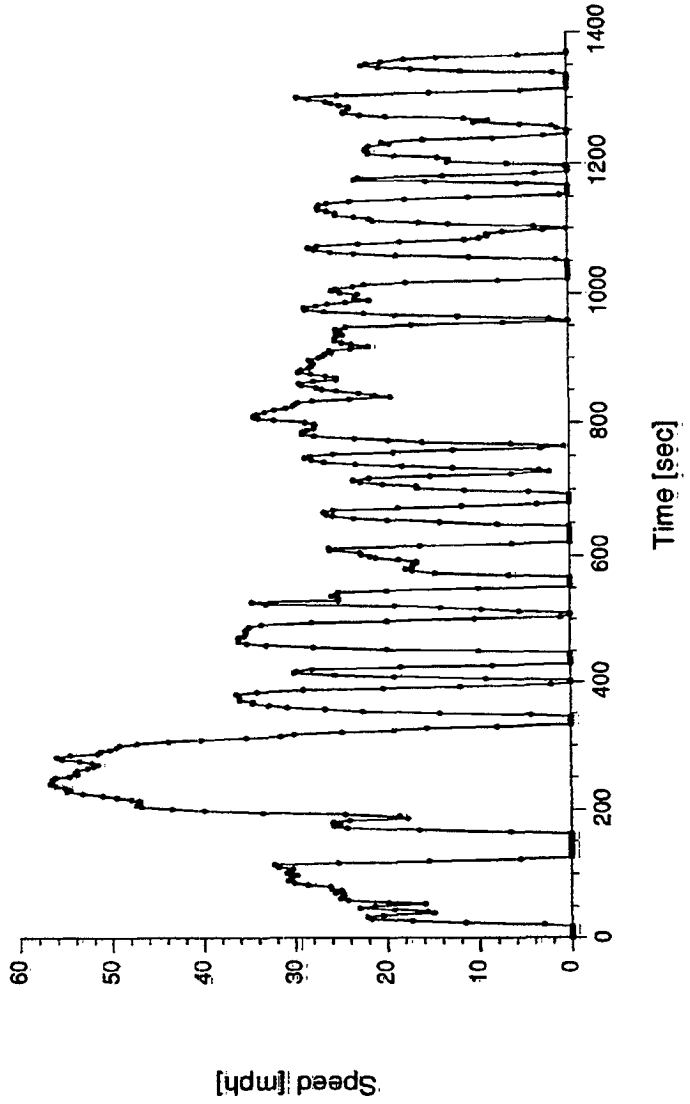

FIGURE IV-8. Graph of U.S. Environmental Protection Agency Urban Dynamometer Driving Schedule

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c. Engine Selection. BNW has identified three manufacturers of light-duty diesel engines that would be acceptable for the proposed studies. Ford Motor Company (Detroit, MI) provides a 7.3-L engine and General Motors Corporation (Detroit, MI) manufactures a 6.5-L engine for their respective lines of pickup trucks. Cummings Diesel (Columbus, IN) also manufactures a light-duty pickup truck engine that is used by Chrysler Corporation (Detroit, MI) in its Dodge pickup trucks. This engine has the lowest particulate emissions of the three and would probably not be our first choice.

At least two engines will be required for the study, one serving as a backup at all times. As presented above, the EPA *Urban Dynamometer Driving Schedule* specifies a 7.5-mile, 23-minute cycle. For the proposed exposures, this will entail the equivalent of approximately 120 miles/day or 31,200 miles/year. Two engines should be adequate to complete the study.

d. Dynamometer System. In order to perform the EPA *Urban Dynamometer Driving Schedule*, a complete vehicle must be run on a chassis dynamometer. However, an accurate simulation of the performance of an engine used in a particular vehicle can be made with a motoring dynamometer. This device controls the load (torque) on the engine during acceleration or deceleration. During deceleration, the drive train is actually turning the engine. There are a number of manufacturers of motoring dynamometers but BNW proposes to utilize a system designed and built by Dyne Systems Co. (Mequon, WI) consisting of a dynamometer with a 220-horse power (hp) absorbing and 200-hp motoring capacity, a direct current (dc) motoring dynamometer controller, and a throttle controller. The system will also have an automated data acquisition and control unit to operate the engine through the required EPA cycles.

Alternatively, a simple absorbing dynamometer system could be used; however, a flywheel system would be necessary to produce the required torque load for the engine. Further, such a system would not provide the load experienced by an engine during deceleration, and would therefore not accurately reproduce the EPA test procedure.

e. Expected Concentrations of Engine Emissions--Particulates and Nitrogen Oxides (NO_x): Diesel engines are manufactured to meet strict and ever-decreasing EPA emission requirements. The current 1993 EPA standard is 0.2 g/mile for particulates, and 1.2 g/mile for oxides of nitrogen. For the toxicity studies, this poses the potential difficulty of producing sufficient diesel particulate while still maintaining safe levels of nitric oxide (NO) in the exposure chambers. In studies on the effects of diesel particulate, the engines employed had much higher particulate emission levels and, consequently, the nitrogen oxides were diluted along with the particulates to manageable concentrations. A chronic diesel exhaust study was performed in our laboratories with General Motors' 4.3-L Oldsmobile engines running at constant speed and torque 20 hours/day, 7 days/week, producing particulate concentrations up to 1.5 mg/m^3. The total NO_x concentrations averaged as high as 7.4 ppm. In a study performed at the Lovelace Institute, a 5.7-L Oldsmobile diesel engine was used to produce particulate concentrations of 7 mg/m^3. Corresponding NO concentrations were 9.82 ppm. This study was performed using a modified EPA driving test procedure. Because the amount of nitrogen oxides produced is largely related to the engine temperature, the ratios of the two measurements (particulates to NO_x) in the studies cannot be compared. Further, the quantity of particulates produced is a function of the driving conditions.

(1) Particulates. To estimate the particulate concentration that will be available and the required engine-emission dilution, a number of assumptions must be made. For the 7.5-mile, 23-minute EPA cycle at 0.2 g/mile average total particulate emissions, the average generation rate of diesel particles would be expected to be 65.6 mg/minute. The instantaneous value would, of course, be constantly changing as the engine cycled. The air flow from the engine would also vary from values as low as 1700 L/minute at idle to as high as 9200 L/minute at full throttle. Choosing an average air flow of 3750 L/minute to match the average particulate generation rate of 65.6 mg/minute, yields an average expected concentration of 17.5 mg/m^3. The proposed high DEE concentration of 10 mg/m^3 should therefore be achievable with up to a twofold dilution of the emissions. If the engine particulate emissions are too low, simple

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adjustments to the fuel pump can be made to increase them, however, fuel pump adjustments also affect the gaseous emissions.

(2) NO_x. Of greater concern than the possibility of insufficient particulate emissions may be the concentrations of NO_x produced by the engine. The engine may produce average concentrations up to 1.2 g/mile. For NO, the principle oxide expected, 1 ppm is equivalent to 1.2 mg/m^3. Applying the same air flow considerations as above suggests that possible average NO concentrations could be as high as 87.6 ppm in undiluted exhaust. This possibility is of concern because the expected dilution will only be a factor of two, resulting in NO concentrations potentially averaging 44 ppm. The threshold limit value (TLV) for NO is only 25 ppm. If the target diesel particulate concentrations are reduced, there will be a corresponding reduction in the NO concentrations. However, the potential for high NO concentrations must be considered in the study design.

f. Control and Characterization of the DEE in the Exposure Units. The exposure concentration will be expressed as the average concentration recorded over each EPA cycle of the engine, approximately every 23 minutes. Adjustments to the exposure concentration will be achieved primarily by adjusting dilution air flow rates. Mass concentrations of DEE in the exposure units will be monitored with a tapered element oscillating microbalance (TEOM| Rupprecht & Patashnick Co., Inc.; Albany, NY) mass monitor and the particle size distribution will be determined with a combination of optical counting devices and cascade impactors, as described in Section IV.C.1.b. Other components of DEE will be measured as described in Section IV.C.1.a.

C. CHARACTERIZATION AND CONTROL OF EXPOSURE SYSTEM

In this proposal BNW has taken the approach that the purpose of chemical-specific measurements is not to exhaustively characterize the chemical composition or physicochemical behavior of DEE or RASS. Rather, the basic approach is to characterize these two very complex mixtures, via the quantitation of judiciously selected marker compounds, such that the composition of the exposure atmospheres can be meaningfully compared to similar studies. The measurements summarized in Table IV-4 represent a limited set of chemical and physical parameters commonly measured to characterize ETS and diesel exhaust. The compositions of the vapor and particulate phases will be characterized by analyzing compounds that are predominantly present in each phase. In addition, the chemical composition of the exposure atmospheres, in terms of both tobacco-specific products and general combustion products, will be performed. The list of analyzed compounds also includes several that are suspected to be the more toxic compounds present in DEE and RASS.

While the proposed chemical-specific characterization of the exposure atmosphere is more extensive than that for some previous studies, this characterization is necessary to allow comparison with previous and future studies and help explain toxicological effects. It is BNW's goal to characterize the exposure atmosphere to meet these updated standards in the current literature. For example, in the case of aromatic amines, PAHs and nitro-PAHs, BNW has expanded the number of specific compounds determined beyond those normally measured as marker compounds. This is expected to involve only a modest increase in cost, while yielding a significant increase in useful information. However, if prestart method development or the subchronic study results show this is not the case, then the number of analytes determined for these classes of compounds will be reduced. Similarly, BNW will also attempt to maximize opportunities to include new measurements that provide significant information about the exposure atmospheres.

In addition to measurements to characterize the chemical composition of the exposure atmosphere, several measurements will be made with the additional purpose of maintaining control of the exposure atmosphere concentration. The concentration of the aerosol will be controlled primarily by frequent, nonchemical-specific measurements of TPM. An on-line, chemical-specific measurement of CO will also be performed. The on-line CO measurements will provide the exposure operator with information needed to

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control the exposure concentrations. Furthermore, it is essential that the animals are not exposed to excessive CO levels.

TABLE IV-4. Chemical-Specific Characterization of the Exposure Atmospheres

Measurement	Method	Frequency	Samples
TPM	Gravimetric	Daily	DEE, RASS
UV/TPM	UV-spectrophometry	2-3/week	DEE, RASS
CO	Nondispersive Infrared	Continuously	DEE, RASS, FA
CO_2	Nondispersive Infrared	Weekly	DEE, RASS
Nicotine/3-ethanyl-pyridine	GC/MS	Weekly	RASS, FA
NO/NO_x	Chemiluminescence	Weekly	DEE, RASS
Trace acids	Ion chromatography	Quarterly	DEE, RASS
Aldehydes	HPLC	Monthly	DEE, RASS, FA
Solanesol	HPLC	Monthly	RASS
Benzene, isoprene, 1,3-butadiene	GC/MS	Monthly	DEE, RASS
Nitrosamines	HPLC/TEA	Quarterly	DEE, RASS
Catechol and other phenols	HPLC	Monthly	DEE, RASS
Selected PAHs	GC/MS	Monthly	DEE, RASS, FA
Nitro-PAHs	HPLC/TEA	Monthly	DEE, RASS, FA
Aniline, 2-aminonapthalene and 4-aminobiphenyl	GC/MS	Monthly	RASS
Heavy metals	ICP-MS	Every 6 months	DEE, RASS, FA
Sulfur dioxide	UV-fluorescence	Monthly	DEE, FA
Total hydrocarbons	FID	Monthly	DEE

KEY: CO = Carbon monoxide; CO_2 = Carbon dioxide; DEE = Diesel engine exhaust; FA = Filtered air; FID = Flame ionization detector; GC = Gas chromatography; HPLC = High-performance liquid chromatography; ICP = Inductively coupled plasma; MS = Mass spectroscopy; PAH = Polycyclic aromatic hydrocarbons; PM = Particulate matter; RASS = Room-aged sidestream cigarette smoke; TEA = Thermal energy analyzer; TPM = Total particulate matter; UV = Ultraviolet

The relationship between TPM, ultraviolet particulate matter (UV-PM), and other frequently determined parameters will be analyzed for trends as the data are obtained. Changes in the relationship between these parameters may indicate that changes are occurring in test atmosphere composition as a result of changes in the generation or delivery systems. The appropriate corrections will then be made to ensure proper control of exposure atmosphere composition and concentration.

1. Methods

a. Chemical Characterization of RASS and DEE Test Articles

(1) TPM and UV-PM. TPM, which includes total solid and liquid aerosols, is the primary controlling parameter for RASS and DEE exposures. BNW anticipates that the proposed generation techniques will result in a TPM particle size for the RASS and DEE exposures that will consist primarily of respirable, suspended particles.

Duplicate filters will be collected at 0.5 L/minute at selected nose ports of each exposure unit for the full exposure period (6 hours plus T_{90}). After filters are equilibrated to a constant temperature and humidity, they will be weighed on a calibrated microbalance. TPM will be defined as the weight increase of the standard glass fiber Cambridge filter (Guerin et al., 1992) following sample collection.

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The UV-PM assay is a method designed to selectively determine the concentration of aromatic compounds on respirable suspended particles in samples originating from ETS, and is designed to be used in tandem with gravimetric TPM measurements. The UV-PM assay involves collecting particles on filters, extracting the filters with methanol, and analyzing the solution spectrophotometrically for UV light absorbance at 325 nm. Standards are either prepared from gravimetric samples of RASS or DEE, or from a surrogate standard material.

The UV-PM assay has two purposes in these studies: 1) determining the extinction coefficient of the generated RASS and DEE will provide information on the chemical nature of the generated aerosols. The UV-PM concentration is believed to be proportional to the concentration of single- and multi-ring aromatic compounds present in the aerosol. These aromatic compounds, in addition to azo- and polyunsaturated compounds, are known to absorb strongly at UV wavelengths. 2) The UV-PM assay enables quantification of filter samples containing RASS and DEE below the limit of quantitation of the gravimetric assay. The UV/PM assay can be validated against gravimetric analysis of TPM samples that can be accurately weighed.

Sampling. Since the collection medium and sample size can both influence the UV-PM assay and gravimetric TPM measurements, the filter medium must trap a high percentage of particles and allow determination of a stable, accurate weight. In addition, it must also give reproducible, efficient recovery of the sample in the solvent for an accurate UV-PM determination. Because RASS and DEE samples may contain particles composed of semivolatile compounds, large sample columns may result in partial evaporation and produce results that underestimate the TPM. To avoid these errors, experiments involving different sampling times and volumes will be performed during the method development phase to validate the UV-PM assay method. Short-term samples of RASS and DEE will be collected using the TEOM monitor to observe the mass lost by evaporation of volatile materials. If a consistent and predictable loss is observed, the TPM filter samples can be corrected for this loss.

The estimated extinction coefficient for PM is 0.0060 to 0.0063 absorbance units (AU)-ml/μg (Rando et al., 1992). DEE is expected to have a similar or higher extinction coefficient due to its relatively high concentration of polycyclic aromatic compounds. Given these extinction coefficients, samples containing as little as 3 μg should permit accurate analyses.

Samples will be collected on 0.2-μm-pore-diameter Teflon membrane filters (Fluoropore FALP 02500; Millipore Corporation, Bedford, MA) from one port of each exposure unit at a flow rate of ~0.5 L/minute for up to 6 hours. This will allow more than adequate sensitivity for UV-PM measurements at the expected concentrations. Gravimetric calibration samples will be obtained from the distribution line at flows of 0.5 to 2 L/minute to trap sufficient TPM to allow an accurate mass determination.

Analytical Methodology. The analytical procedure described below is based on the work of Conner et al. (1990), who found the method to give good collection efficiency, low breakthrough, and little or no sample evaporation for sample volumes up to ~240 L.

Filters will be conditioned at ~22°C and 60% RH before and after weighing with a microbalance to obtain the tare and final masses. Sample filters will be placed in clean, labeled, ~24-ml glass bottles with Teflon-lined caps. The samples will be extracted by adding 2.0 to 20.0 ml of methanol, followed by 30 minutes of orbital shaking. Tests will be performed during methods development to ensure complete extraction is achieved using this technique. Approximately ~1.5 ml of extract will be transferred to labeled automatic liquid sample (ALS) vials and analyzed by flow injection analysis with a HPLC UV/visible detector.

Calibration will be accomplished by weighing filter samples collected from the distribution lines of either the RASS or DEE exposure systems. These samples, with a known mass of TPM, will be extracted and serial dilution of the extracts will be made. Using these as standards, a calibration curve of peak area

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(from flow injection analysis) versus concentration (microgram/milliliter) will be prepared and used to analyze samples taken from the exposure units.

Standards and samples will be analyzed using flow-injection analysis with automated sample analysis and computerized calibration curves. The flow-injection analysis parameters are described in Table IV-5. Analysis time is estimated to be less than 30 seconds/sample or standard.

TABLE IV-5. Flow-Injection Analysis Parameters for UV Particulate Matter

Detector	ABI 785A ultraviolet/visible
Autosampler	Hewlett-Packard (HP)-1050
Data Acquisition Hardware	Gateway 2000
Data Acquisition Software	HP high-performance liquid chromatography chem-station (DOS Series), version A.01.03
Buffer Column	~300 cm, 0.2 mm stainless steel
Mobile Phase	100% methanol
Injection Volume	100 µl
Flow Rate	1 ml/minute
Analytical Wavelength	325 nm
Calibration	External calibration method

In addition, the molar extinction coefficient for RASS and DEE TPM will be evaluated. The absorbance of filter extracts containing known amounts of TPM will be measured at 325 nm using conventional spectrophotometry.

The results of samples collected and analyzed for TPM by UV absorbance will be reported in micrograms/milliliter. UV-PM results will be multiplied by the total extraction volume to obtain micrograms/sample. This will be divided by the volume of the exposure atmosphere sampled to obtain TPM concentrations in milligrams/cubic meter. Results obtained by UV-PM will be compared to the TPM concentration determined by weighing Cambridge filter pads and the results from the TEOM monitor.

(2) Carbon Monoxide (CO). Carbon monoxide is a commonly recognized toxin that is lethal at very high concentrations and contributes to cardiovascular and other health effects with prolonged exposure. Popular U.S. commercial filter-tipped cigarettes deliver approximately 15 mg of CO in mainstream smoke and an additional 50 mg in sidestream smoke (Guerin et al., 1992). Carbon monoxide emission in DEE from light-duty trucks has been found to be about 1270 mg/mile (International Agency for Research on Cancer [IARC], 1989).

Analytical Methodology. BNW proposes using a Horiba CO monitor (Model 350-E, Horiba Instruments, Inc.; Irvine, CA), which is designed for reliable, continuous, unattended measurements of CO. The Model 350-E employs nondispersive infrared absorption to determine the presence of CO. This instrument exceeds all performance specifications made by EPA and the European Environmental Testing Agency. The

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output of the CO monitors will be interfaced to the main data acquisition and control system by a dedicated computer running proprietary software developed by BNW.

The Horiba Model 350-E has a lower limit of detection of 0.06 ppm for CO and electronic noise within 0.03 ppm. The instrument has a very high specificity for CO; the only reported interference being water (~0.4 ppm CO at 100% RH). BNW will maintain the exposure atmosphere at ~60% RH; thus there will not be significant interference resulting from the presence of water vapor in the system. The working range of the Horiba Model 350-E is 0 to 100 ppm CO, which BNW anticipates will be adequate.

Because the mass concentration of DEE is continually changing as the engine is cycled, one CO monitor will be required to measure the CO concentration in the DEE exposure unit pair. This CO monitor will report the average CO concentration during the 23-minute engine cycle. A second CO monitor will be multiplexed to measure the CO concentration in each RASS exposure unit pair, the filtered-air unit pair, and the RASS aging room.

The monitors will be calibrated against commercially certified CO gas standards. The calibration will be verified during routine operations by measuring an on-line gas standard containing a known concentration of CO.

(3) Carbon Dioxide (CO_2) Determination. Carbon dioxide, produced by the combustion process, is a major constituent of ETS (Greyson, 1990) and DEE (IARC, 1989).

Analytical Methodology. BNW proposes using a Horiba Model VIA-510 monitor, which is designed for reliable, continuous, unattended measurements of CO_2. This model employs nondispersive infrared absorption and is highly specific for CO_2.

The Horiba Model VIA-510 has a lower limit of detection of ~2 ppm for CO_2. BNW estimates that the lowest concentration of CO_2 to be measured will be ~15 ppm over the ambient concentration of 330 ppm. Thus the instrument will have acceptable sensitivity for this study. The VIA-510 detector has the unique capability of measuring sample gases containing particularly high levels of interfering substances. The working range of the Model VIA-510 is 0 to 100% CO_2, although it is also equipped with specific ranges to improve sensitivity and accuracy.

The monitor will be calibrated monthly using certified standard gases encompassing the expected concentration range. A certified standard of CO_2 in nitrogen will be sampled prior to routine measurements to determine detector drift.

(4) Nicotine/3-Ethenylpyridine. Nicotine and its pyrolysis product, 3-ethenylpyridine, have been widely used as markers for ETS in the assessment of indoor air quality. ETS is the major source for these compounds in indoor air, which makes them selective markers for concentrations of vapor-phase components of ETS. Unlike nicotine, the fate of 3-ethenylpyridine has been shown to closely mimic that of other volatile organic compounds produced from tobacco smoke (Eatough et al., 1989; Heavner et al., 1992; Nelson et al., 1992). Thus, monitoring 3-ethenylpyridine concentrations will provide a marker for the concentration of volatile components of RASS.

Research on tobacco smoke usually includes measurements of nicotine, and considerable data are available on the relation between smoke composition and nicotine concentrations. Nicotine in ETS is predominantly present in the vapor phase as the free base. Recent studies have shown that nicotine in the vapor phase is rapidly depleted from the field environment, as well as from test atmospheres, due to surface adsorption (Guerin et al., 1992), leading many researchers to question the usefulness of using nicotine as a marker for volatile organics in ETS. However, monitoring nicotine concentrations will serve to relate the proposed study to previous research.

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Sampling. XAD-4 sorbent tubes (high-surface-area styrene-divinylbenzene copolymer resin) will be used to collect both nicotine and 3-ethenylpyridine from the RASS exposure atmospheres. Exposure atmosphere samples will be drawn through the sampling tubes for 10 minutes each at a flow rate of ~0.25 L/minute. For RASS exposures ranging from 1 to 10 μg/L, the expected amount of nicotine collected will range from 250 to 2500 ng; the amount of 3-ethenylpyridine is expected to range from 25 to 250 ng.

Analytical Methodology. Loss of nicotine is frequently observed on the walls of sample containers and sample manipulation accessories used in collection and analysis procedures. To minimize this adsorption, a strong base (triethylamine) will be added to the extraction solvent to preferentially react with the acidic sites on the glassware (Ogden et al., 1989). A variety of studies have been performed with triethylamine-spiked extraction solvent, showing good extraction efficiency for 3-ethenylpyridine. After collection, each bed of the sample tubes will be desorbed with 1.0 ml of 0.01% triethylamine in ethylacetate. The ethylacetate solution will contain 1000 ng/ml nicotine (methyl-D3) and 100 ng/ml 3-ethenylpyridine (ethenyl-D3) as isotopically labeled internal standards.

The samples will be analyzed by GC/MS using an HP-5971 MSD system operated in the selected ion monitoring mode. The instrument will be calibrated against gravimetrically prepared standards of nicotine and 3-ethenylpyridine. Standards will be freshly prepared for each analysis set. A 5% diphenyl to 95% dimethyl polysiloxane capillary column will be used for chromatographic separation.

Samples will be collected twice each week for nicotine and 3-ethenylpyridine determinations. Samples will be collected shortly after the start of exposure and again shortly before the end of exposure from each of the three concentrations of RASS test atmospheres, as well as from the filtered-air atmosphere. The sample tubes from both days of sampling will be saved and analyzed as one set within the week of collection; the tubes will be capped and kept at ~5°C until analysis.

The XAD-4 sample tubes contain two separate beds of sorbent. During exposure generation testing, exposure atmosphere samples will be collected and both beds will be analyzed for nicotine and 3-ethenylpyridine. The collection efficiency will be assessed for these compounds by comparing breakthrough from the initial sorbent bed to the backup sorbent bed. BNW expects >95% collection efficiency using the XAD-4 sample tubes. Alternative collection media will be investigated if significant breakthrough is encountered.

For samples collected for exposure assessment, the sorbent beds will be desorbed and analyzed within 5 days after sample collection. During generation testing, analyses will be performed to assure that degradation of the analytes does not occur on the sorbent bed prior to desorbtion. Duplicate sorbent tubes will be collected from each of the RASS exposure units. One set of tubes will be immediately desorbed and analyzed, while the duplicate will be capped and stored for 1 week before being desorbed and analyzed. If significant degradation is noted, the sample tubes will be desorbed and analyzed immediately after sample collection for exposure assessment.

During method validation, standards will be prepared with and without XAD-4 sorbent to assess analytical interferences and sample loss arising from the sorbent material.

(5) Nitrogen Oxides and Trace Acids. The oxides of nitrogen (NO_x), are principally formed by the combustion of nitrogen-containing constituents of fuels and by the high-temperature oxidation of nitrogen. Nitrogen dioxide (NO_2) and nitric oxide (NO) comprise more than 90% of the NO_x in cigarette smoke (Guerin et al., 1992). It is well established that NO and NO_2 can be used as markers for engine exhaust (IARC, 1989).

Analytical Methodology for NO and NO_x. BNW proposes the use of the LMA-3 NO_2 analyzer (Scintrex, Ontario, Canada) to measure NO_2 concentrations in RASS and DEE. This analyzer offers sensitive, direct detection of NO_2 based on the chemiluminescence resulting from NO_2 reaction with an aqueous

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luminal solution. Operating this analyzer with an LNC-3 Luminox NO converter accessory (Scintrex, Ontario, Canada) will permit sequential detection of NO_2 and NO_x (NO_2 + NO) via the conversion of NO to NO_2.

An additional advantage of this instrument is its highly specific response to NO_2. The response of the analyzer is insensitive to common trace atmospheric gases such as nitric acid, ammonia, sulfur dioxide (SO_2), formaldehyde, CO_2 and CO. The LNC-3 NO converter also possesses operational characteristics that permit interference-free measurement of NO_x. In contrast, NO_x converters associated with NO chemiluminescent analyzers are subject to the positive interference that results when other nitrogen-containing trace gases are converted to NO. The efficiency of the LNC-3 NO converter must, however, be determined at the time of calibration in order to document stable converter performance. The vendor-declared detection limit of the LMA-3 analyzer is <0.01 ppb NO_2. The response is linear above ~2 ppb.

The analyzer will be calibrated using a certified standard NO_2 permeation tube with a dynamic calibrator. Calibration mixtures over the range of concentrations encountered in the test atmospheres will be generated by adding metered amounts of purified air to the gas stream used to flush the thermally controlled permeation chamber. A certified gas mixture of NO in nitrogen will be used to confirm the effeciency and stability of NO conversion. A certified standard of NO and NO_2 in nitrogen will be sampled before exposure atmosphere sampling.

Analytical Methodology for Nitrous Acid (HNO_2) and Nitric Acid (HNO_3). Periodic measurement of the inorganic acids HNO_2 and HNO_3 is also recommended to fully characterize the test atmospheres. Nitric acid is produced from NO_x in the open atmosphere and is an important contributor to atmospheric acidity. Nitric acid has also been measured in both DEE and in ETS, although the concentrations in ETS have been reported to be more than an order of magnitude less than that of HNO_2, the major inorganic acid component (Eatough et al., 1989).

Acidic gases will be collected by passing a metered flow of the test atmosphere through an annular denuder. This device collects the analyte of interest on chemically treated walls of concentric glass tubes while allowing the passage of potentially interfering aerosol particles. Acidic gas collection is achieved with etched surfaces coated with sodium carbonate (Na_2CO_3)/glycerine. At the end of the sampling period, the collected acids will be removed by extraction and measured as nitrite and nitrate ions by ion chromatography. In addition to minimizing potential interference from particulate nitrate, the proposed sampling method also collects SO_2, providing an independent determination of SO_2.

(6) Formaldehyde, Acrolein, and Other Aldehydes. At least 20 aldehydes have been identified in the vapor phase of cigarette smoke, the most abundant being formaldehyde (3 to 40 μg/cigarette), acetaldehyde (519 to 1144 μg/cigarette), propionaldehyde (25 to 116 μg/cigarette), and acrolein (11 to 228 μg/cigarette; IARC, 1985). Several of these compounds, especially formaldehyde and acrolein, are toxic to cilia. They can inhibit lung clearance after inhalation. Formaldehyde induces nasal carcinoma in rats (IARC, 1985). These compounds are also the most prevalent aldehydes in DEE (Lipari and Swarin, 1982). The estimated concentrations of these aldehydes in ~1 mg/m^3 TPM RASS and 10 mg/^{3} TPM DEE are listed in Table IV-6.

Because formaldehyde, acrolein, and other aldehydes are reactive and are present at low concentrations in the vapor phase of smoke, current sampling practices rely heavily on derivatization for accurate analysis. The measurement of aldehydes is subject to error as the result of interferences, particularly from ozone.

Sampling. The most common methods of aldehyde analysis involve derivatization with 2,4-dinitrophenylhydrazine (DNPH). Samples are generally collected in glass impingers containing a solvent and the derivatizing agent. To increase sample throughput and avoid problems with DNPH contamination, BNW proposes sample collection using DNPH-coated silica cartridges (Sep-Pak Silica® DNPH cartridges; Waters, Milford, MA), incorporating an ozone scrubber to reduce ozone interferences observed with solid-phase sam-

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pling. Samples will be collected from a nose port of each exposure unit at a flow rate of ~0.25 L/minute for up to 6 hours, if needed, which will allow more than adequate sensitivity at the expected concentrations.

TABLE IV-6. Aldehyde Concentrations in Room-Aged Sidestream Cigarette Smoke (RASS) and in Diesel Engine Exhaust (DEE)

Compound	Approximate Concentration in RASS (µg/L)	Approximate Concentration of DEE (ppm)
Formaldehyde	0.05 to 0.5	0.25
Acrolein	0.08 to 0.3	0.05
Acetaldehyde	0.5	0.05
Propionaldehyde	0.05	0.03

To avoid contamination, unopened sample pouches will be stored at ~3°C until ready for use. Since background levels of hydrazone derivatives increase slightly with time and temperature, cartridges stored at room temperature in sealed pouches for periods over 1 week will be tested prior to use.

Analytical Methodology. Samples will be collected directly on DNPH-coated silica sample tubes. Immediately after sample collection, tubes will be capped tightly and placed in a labeled sample pouch. The samples will be extracted with internal standard (cyclohexanone) in 1 ml of acetonitrile over 1 minute, followed by 3 ml of acetonitrile over 1 minute into a 5-ml volumetric flask. The extract will be diluted to a final volume of 5 ml. After diluting to volume, the samples will be mixed and ~1.5 ml will be transferred to labeled ALS vials and analyzed by HPLC (Table IV-7).

The accuracy of the solid-phase sample tubes and incorporated ozone scrubber will be verified using the impinger method before the start of the study. Glass impingers containing acetonitrile and DNPH will also be used to collect the aldehydes. The impingers will be prepared the same day the sample analysis is to take place. All stock solutions of DNPH will be kept isolated and under vacuum until the day of analysis. Once impingers are prepared, they will be kept capped until immediately prior to sampling. Caps will be replaced immediately after sampling. A known volume of internal standard will be added to each impinger after sampling. The impingers will then be mixed well and ~1.5 ml will be transferred to labeled ALS vials and analyzed by HPLC (Table IV-7).

Validation and recovery of both the solid-phase sample tubes and the impinger methods will be accomplished by spiking the sample devices with known concentrations of the aldehydes. Standards will be prepared with the same internal standard to be used in sampling. The range of standard concentrations bracket the aldehyde concentrations expected in samples for both RASS and DEE.

Initial sample analysis of RASS cartridge or impinger samples will be used to screen the samples for the presence of other aldehydes. These results will allow a general characterization of aldehydes and ketones present in the RASS and DEE.

(7) Solanesol. Solanesol, a high-molecular-weight trisesquiterpenoid alcohol, is associated exclusively with the particulate fraction of ETS. It is very specific to tobacco smoke and is a reliable tracer for respirable suspended particles attributable to ETS (Ogden and Maiolo, 1992).

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TABLE IV-7. High-Performance Liquid Chromatography Analysis Parameters for 2,4-Dinitrophenylhydrazine Derivitives of Aldehydes

Detector	ABI 785A ultraviolet/visible
Autosampler	Hewlett-Packard (HP)-1050
Data Acquisition Hardware	Gateway 2000
Data Acquisition Software	HP High-performance liquid chromatography chemstation (DOS Series), version A.01.03
Analytical Column	Nova Pak C18, 3.9 mm x 150 mm; Part #86344
Mobile Phase	65% ultra-pure water; 30% acetonitrile; 5% tetrahydrofuran (uninhibited)
Injection Volume	10 µl
Flow Rate	1.5 ml/minute
Analytical Wavelength	360 nm
Internal Standard	Cyclohexanone; 99.8% (Aldrich; Catalog #C10,218-0)
Formaldehyde Standard	ACS Reagent; 37 wt% (Aldrich; Catalog #25254-9); Retention Time (RT) = ~3.2 minutes
Acrolein Standard	97% (Aldrich; Catalog #11022-1); RT = ~6.0 minutes
Acetaldehyde Standard	99% (Aldrich; Catalog #11007-8); RT = ~4.2 minutes
Propionaldehyde Standard	97% (Aldrich; Catalog #P5145-1); RT = ~6.7 minutes
2,4-Dinitrophenylhydrazine	70% (Aldrich; Catalog #D19930-3)

Solanesol is the most abundant terpenoid and acyclic isoprenoid found in the lipid fraction of tobacco. Isoprenoids are present in neutral subfractions of tobacco smoke condensate that have been reported as carcinogenic and tumor-promoting on mouse skin. Pyrolysis of tobacco extract, highly enriched with solanesol and its esters gave rise to the highest amounts of PAHs determined of any tobacco extract fractions or subfractions (IARC, 1985).

Sampling. Samples will be collected on 0.2-µm-pore-diameter membrane filters (Fluoropore FGLP 02500, Millipore Corp., Bedford, MA) from each exposure unit at a flow rate of ~1 to 4 L/minute for up to 6 hours. This should allow adequate sensitivity at the expected concentrations. The estimated concentration levels of solanesol in ~1 µg/L TPM RASS is ~0.05 µg/L.

Analytical Methodology. Recently Ogden and Maiolo (1992) compared GC and HPLC methodologies to determine solanesol in ETS. The GC procedure requires derivatization and manual on-column injection using a fused silica needle. The GC procedure was labor intensive and sample workup was inconsistent with companion analysis of the filter extracts for particulate matter by UV absorbance or fluorescence.

The HPLC method requires detection at 205 nm with a mobile phase of 95%:5% acetonitrile:methanol to achieve adequate baseline stability. Using a 100-µl injection volume provided an HPLC

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detection limit comparable to that of GC. The HPLC sample workup was simpler and provided an extract that was compatible with determining UV and fluorescent particulate matter. On the basis of these investigations, BNW plans to implement the HPLC method described by Ogden and Maiolo (1992) to determine solanesol content of the RASS particulate mater.

Filter samples will be placed in clean glass ~70 ml bottles immediately after collection. The samples will be extracted by adding ~3 ml of methanol, followed by sonication for 30 minutes. Approximately ~1.5 ml of extract will be transferred to labeled ALS vials and analyzed by HPLC as indicated in Table IV-8.

TABLE IV-8. Proposed High-Performance Liquid Chromatography Parameters for Solanesol Analysis

Detector	ABI 785A ultraviolet/visible
Autosampler	Hewlett-Packard (HP)-1050
Data Acquisition Hardware	Gateway 2000
Data Acquition Software	HP High-performance liquid chromatography chemstation (DOS Series), version A.01.03
Analytical Column	C8, 5 μm, 25 cm x 4.6 mm (Astec, Inc.; Whippany, NJ)
Mobile Phase	95% acetonitrile; 5% methanol
Injection Volume	100 μl
Flow Rate	1.2 ml/minute
Analytical Wavelength	205 nm
Calibration	External calibration method
Solanesol Standard	90+% (Sigma; Catalog #S8754); Retention Time = ~9.1 minutes

Validation and recovery of the solanesol sampling method will be accomplished by spiking the sample device with known concentrations of solanesol standards. The standards will be prepared gravimetrically and spiked onto the same filter medium as the samples. Standards will then be extracted as above. The range of standards will bracket the solanesol concentrations expected in RASS.

The most serious drawback to HPLC analysis of solanesol is the lack of a solanesol chromophore. It was found that UV detection is possible only at 205 nm, where nearly all organic compounds absorb. By using acetonitrile as the mobile phase, however, baseline stability can be achieved. It was also found that a UV detector with a deuterium lamp was ~50 times more sensitive at 205 nm than a UV detector with a xenon lamp. Chromatographic parameters will be modified to achieve the best possible detection and separation of solanesol. Using this method, Ogden and Maiolo (1992) indicated a detection limit of 0.02 μg with recovery of ~90%.

(8) Benzene, Isoprene, 1,3-Butadiene. The compound 1,3-butadiene has been shown to be a carcinogen in rats and mice (Huff et al., 1985). Benzene is a recognized human carcinogen (IARC, 1989), and isoprene is currently undergoing carcinogenicity testing in rodents. It has been suggested

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that isoprene might be utilized as a tobacco smoke tracer for unsaturated aliphatic hydrocarbons (Laforth et al., 1989).

This procedure is specifically designed to quantify 1,3-butadiene, isoprene, and benzene at concentrations of ~0.01-0.3 μg/L using a GC/MS analysis method. For a RASS concentration of 1 μg TPM/L, these compounds are expected to be present at approximately the following concentrations: 1,3-butadiene, ~0.03 μg/L; isoprene, ~0.15 μg/L; and benzene, ~0.04 μg/L.

Sampling. BNW proposes to use ORBO-32 charcoal sampling tubes (Supelco, Bellefonte, PA) for collecting RASS samples. BNW has extensively characterized the precision, accuracy, recovery, and breakthrough of isoprene collected, and has found them to be exceptionally well suited to this task. BNW anticipates that these tubes will also be suitable for collecting benzene and 1,3-butadiene.

Before the start of the study, breakthrough will be determined by preparing semi-quantitative gas bag standards of the components of interest. The gas bag standard preparations will contain approximately the same total amount of these compounds that will be collected from the RASS exposure. Breakthrough will be measured by comparing the amount of the compound found in the secondary bed of the sample tube to that found in the primary bed.

Analytical Methodology. BNW proposes using an HP-5890 gas chromatograph equipped with an HP-5971 MSD to analyze tube extracts. A J&W DB-1 capillary column (or equivalent) will provide separation of the benzene, isoprene, and 1,3-butadiene. Using this system, the limit of quantitation for these compounds is expected to be ~5 to 50 pg/μl.

Validation and recovery of the sampling method will be accomplished by spiking the sampling device with known concentrations of benzene, isoprene, and 1,3-butadiene. Standards of these chemicals will be prepared in the concentration range expected in the RASS samples. An internal standard will be used to compensate for variation in injection volume. These standards will be prepared with and without the charcoal bed from an ORBO-32 sampling tube. Linearity, recovery, precision, and accuracy will be determined from these standards.

During prestart testing, BNW will also collect samples from the distribution line and from one port of the exposure unit to characterize background levels of these compounds before tobacco smoke is introduced into the exposure system. Samples will be analyzed by acquisition of the full mass spectrum and a library search will be conducted on the mass spectrum from all major peaks. These measures will allow BNW to determine whether any other compounds identified by the library search need to be investigated further or be included in the analysis procedure for the chronic study.

(9) Nitrosamines. N-nitrosamines are of particular interest because they are quite biologically active. Tobacco smoking is the only known source for nitrosamines in nonoccupational indoor air, where they are found in significantly greater amounts in sidestream smoke than in mainstream smoke (Guerin et al., 1992). The major tobacco-specific nitrosamines are generally recognized as NNN and NNK, which originate from nicotine (Brunnemann et al., 1992; Hecht et al., 1992; Hoffman and Hecht, 1985).

Sidestream smoke also contains several volatile nitrosamines. N-nitrosodimethylamine (DMNA), N-nitrosodiethylamine (DENA), and N-nitrosopyrrolidine (NPYR) are the volatile nitrosamines formed in the greatest amounts and are typically selected for routine quantitative measurements (Brunnemann et al., 1980; Guerin et al., 1992). The volatile nitrosamines can originate from protein pyrolysis, as well as from other processes, resulting in their presence in a wide variety of consumer products. They are also present at detectable quantities in ambient air (Brunnemann et al., 1980; Guerin et al., 1992; IARC, 1985). Nitrosamines have also been reported in diesel engine crankcase emissions (Goff et al., 1980).

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The procedure implemented by BNW will be designed to quantify NNN, NNK, DENA, DMNA, and NPYR at concentrations of ~5 to 50 ng/m^3. For a RASS concentration of 1 mg/^{3}, these compounds are expected to be present at approximately the following concentrations: NNN, ~10 ng/m^3; NNK, ~20 ng/m^3; DMNA, ~20 ng/m^3; and NPYR, ~10 ng/m^3.

Sampling. Nitrosation of amines occurs rapidly in the presence of NO_x. To accurately quantify the concentration of a nitrosamine, nitrosation must be prevented within the collection medium during and after sampling. Initially, BNW will collect nitrosamines on potassium-bisulfate-treated Cambridge filters (Brunnemann et al., 1992) to gain sensitivity and possibly reduce sample-preparation time. Another common method to block nitrosation involves collecting the sample in a glass bubbler or impinger tube containing an ascorbic acid solution buffered at an approximate pH of 4 to 4.5 (Brunnemann et al., 1992). BNW intends to use this sample-collection method as an alternative if problems are encountered with collection on filters.

Analytical Methodology. BNW proposes to procure a thermal energy analyzer (TEA; Thermedics, Model 502, Woburn, MA), which can be coupled to either GC or HPLC for detection. The TEA detector is highly selective for nitrosamines and the sensitivity is expected to be ~50 to 150 pg.

Although recently published methods indicate good results can be obtained for determining tobacco-specific nitrosamines using GC with thermal desorption (Brunnemann et al., 1992), BNW prefers using a narrow-bore HPLC for our initial attempts because of our previous experience with NNK analysis by HPLC. This approach avoids problems that may result from decomposition in the heated zones of the GC or thermal desorber. In the event that adequate performance cannot be achieved with HPLC, BNW will implement the thermal desorption/GC method of Brunnemann et al. (1992).

BNW will evaluate the collection efficiency of potassium-bisulfate-treated Cambridge filters for nitrosamines during prestart testing. A filter will be spiked with known concentrations of the target nitrosamines listed above. Air will then be drawn through the filter in the same manner the samples are collected from the exposure system. These filter samples will then be analyzed for recovery, precision, and accuracy relative to gravimetrically prepared standards. If BNW demonstrates sufficient collection efficiency of these treated filters, samples will be collected from the distribution line using both treated filters and bubblers containing a buffered solution of ascorbic acid. This latter method of collection has been shown to block nitrosation of amines and thus provide a reference point for the treated-filter method.

A nontobacco-related nitrosamine will be used as an internal standard to correct for preparation errors. Another internal standard will be added after sample preparation to compensate for variations in injection volume and detector response.

(10) Catechol and Other Phenols. Over 200 semivolatile phenols have been identified in cigarette smoke, including various polyphenols (IARC, 1985). The more prevalent phenols and polyphenols found in cigarette smoke are shown in Figure IV-9. Phenol is the most prevalent of these compounds in mainstream smoke. The cresol isomers, xylenols, 2-methoxyphenol, and 4-vinylguaiacol are found at lower concentrations. Catechol is the major hydroxyphenol identified in cigarette smoke, with smaller amounts of cresols (three and four isomers), 4-ethylcatechol, and 4-vinylcatechol. The primary precursors of volatile phenols and catechols are believed to be glucose, pectins, rutins, polysaccharides and polyphenols.

The method described below allows the determination of hydroquinone, resorcinol, catechol, phenol, m+p-cresol, and o-creosol. Because of their similar chemical characteristics and ability to determine them with a common analytical method, BNW proposes to include them in the catechol analyses as described below.

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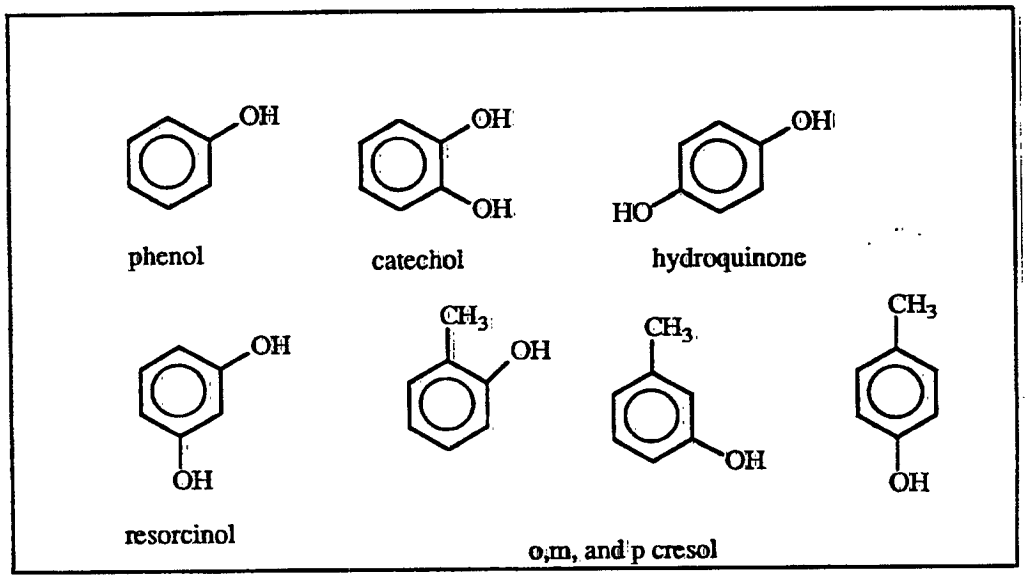

FIGURE IV-9. Tobacco-Related Phenols and Catechols

The method described below uses the sensitivity of the fluorescence detector to allow detection of low phenol/catechol concentrations. The method also uses time-programmed selection of wavelengths to allow increased selectivity for the compounds of interest.

The estimated concentrations of catechol and phenol in 1 μg/L of RASS are expected to be ~0.01 μg/L of each component. Hydroquinone, resorcinol, and cresols are expected to be present at approximately one-half to one-fifth the concentration of the phenol. Given these concentrations and an analytical limit of quantification of <50 ng, an elution volume of 15 ml, and an injection volume of 1 ml, a minimum sample size of ~75 L will be required from the 1-μg/L RASS exposure to determine phenol and catechol. Typical samples from the exposure units are expected to be ~180 L. The lower concentrations of the substituted phenols and catechols may prevent accurate determination of these substituted compounds at the lower concentrations of RASS.

Sampling. Samples will either be collected on Cambridge filter pads or in solvent-filled impingers from a port on each exposure unit at a flow rate of ~0.5 L/minute for up to 6 hours (180 L), allowing more than adequate sensitivity at the expected concentrations.

Analytical Methodology. The analytical procedure described below is based on the work of Risner and Cash (1990), who found this method to give good collection efficiency, low breakthrough, and high recovery. Samples collected on Cambridge pads will be extracted with 15 ml of 1% aqueous acetic acid. Impingers will be prepared using 15 ml of 1% aqueous acetic acid. Sample aliquots will be transferred to ALS vials for analysis.

Standards will be prepared gravimetrically in 1% aqueous acetic acid using two gravimetrically prepared stock solutions and highly pure commercially available neat material. Standards and samples will

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be analyzed using HPLC with automated sample analysis (Table IV-9) and computerized calibration curves. Analysis time is estimated to be ~60 minutes/sample or standard.

TABLE IV-9. High-Performance Liquid Chromatography Parameters for Phenol and Catechol Analysis

Detector	Hewlett-Packard (HP)-1046A programmable fluorescence detector
Pump	HP-1050 quaternary
Autosampler	HP-1050 with extended sample loop
Data Acquisition Hardware	Gateway 2000
Data Acquition Software	HP High-performance liquid chromatography chemstation (DOS Series)
Analytical Column	4.6 x 250 mm ODS
Mobile Phase	Gradient of 100% of 1% aqueous acetic acid to 100% of 1% acetic acid in acetonitrile
Injection Volume	1000 µl
Flow Rate	1.5 ml/minute
Analytical Wavelength	Timed program: excitation of 274 to 304 nm; emission: 298 to 338 nm
Calibration	External calibration method

Analytical results will be reported in micrograms/liter and as micrograms of compound/micrograms of RASS.

(11) Selected Polycyclic Aromatic Hydrocarbons (PAHs), Nitropyrene, and Nitro-Polycyclic Hydrocarbons. Polynuclear aromatic compounds (PACs) are a group of highly lipophilic chemicals that occur throughout the environment as pollutants, generally at trace levels. PACs arise from the combustion of organic matter from a wide variety of sources including DEE and mainstream and sidestream cigarette smoke. All classes of PACs are characterized by the polyaromatic structure, but the classes of from the nonpolar PAHs to more polar classes possessing functional groups containing oxygen, sulfur, or nitrogen. These include aldehyde and carboxylic-substituted PAHs; azarenes, amino-PAH, hydroxy-PAH and nitrated-PAH (nitro-PAH). Since the PACs have low vapor pressures, and are generally formed in association with combustion processes that also produce particles, they are predominately found adsorbed on particulate material. Since a large fraction of the PAC is associated with ultra-fine particles, the particle-borne PAHs are respirable (IARC, 1985).

At one time diesel exhaust was believed to be carcinogenic because of the presence of PAHs. It has subsequently been discovered that diesel particle extract contains over 100 nitro-PAHs (Paputa-Peck et al., 1983). Nakagawa and coworkers (1983) found that the dinitropyrene (DNP) isomers contributed 43% of the total mutagenic activity of the crude extracts, whereas 1-nitropyrene (1-NP) was responsible for less

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than 10% of the activity. Thus, although 1-NP is the predominate nitro-PAH, measurement of 1,6- and 1,8-DNP would appear to provide a more complete characterization of the nitro-PAH content of RASS and DEE.

The PAHs and nitro-PAHs selected for analysis are listed in Table IV-10. Part of the rationale for including benzo[a]pyrene (BaP), 1-NP, and the DNP isomers was discussed earlier; i.e., the strong biological activity of these compounds and the widespread use of 1-NP and BaP as markers. Besides BaP, the other specific PAHs in the four- to six-ring size were chosen based on the availability of standards, their biological activity, and their inclusion in several relevant standard reference materials (SRM) available through the National Institute of Standards and Technology. Also, most of the PAHs suspected as carcinogen contain four- to six-rings.

TABLE IV-10. Selected Analytes and Some Standard Reference Materials (SRMs) Containing Polycyclic Aromatic Hydrocarbons (PAHs) and Nitrated Polycyclic Aromatic Hydrocarbons (Nitro-PAHs)

Parent PAH	Molecular Weight	Stable Label	SRM #1650[a] (mg/kg)	SRM #1491[b] (mg/kg)	Nitro-Derivative(s)	SRM #1587[c] (mg/kg)	SRM #1596[d] (mg/kg)
Naphthalene	128.2	Aldrich		10.3	1 and 2-Nitro		
Biphenyl	154.2	Aldrich			4-Nitro		
Acenaphthene	154.2	Supelco		10.89			
Fluorene	166.2	Isotec		10.87	2-Nitro	9.67	
Anthracene	178.2	Aldrich	[0.88]	11.69	9-Nitro	5.01	
Phenanthrene	178.2	Aldrich	[71]	10.48			
Fluoranthene	202.3	Isotec	51	8.84	3-Nitro	9.24	
Pyrene	202.3	Isotec	48	8.81	1-Nitro 1,6-Dinitro 1,8-Dintro	8.95	4.38 4.82 8.9
Benzo[a]anthracene	228.3	Isotec	6.5	5.37	7-Nitro	9.27	
Chrysene	228.3	Aldrich	[22]	10.5	6-Nitro	8.13	
Benzo[k]fluoranthene	252.2		[2.1]	8.33			
Benzo[b]fluoranthene	252.2		7.85				
Benzo[a]pyrene	252.3		1.2	10.14	6-Nitro	6.1	
Benzo[ghi]perylene	276.3		2.4	7.9			

[a]SRM#1650 is diesel particulate matter and contains 19 mg/kg of 1-nitropyrene in addition to the listed PAHs. Values in brackets are uncertified (May et al., 1992).
[b]SRM#1491 is aromatic hydrocarbons in hexane/toluene
[c]SRM#1587 is nitrated PAHs in methanol
[d]SRM#1596 is dinitropyrene isomers and 1-nitropyrene in methylene chloride and also contains 1,3-dinitropyrene at 8.9 mg/kg

One reason for including pyrene is its role as a precursor to 1-NP and the DNPs. Including 1-NP and the DNPs will allow the assessment of relative amounts of these compounds and should allow determination of the relative degree of nitration of parent PAH throughout the study. Similarly, both 7-nitrobenzo[a]-

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anthracene and 6-nitrochrysene have been reported in diesel exhaust (Niles and Tan, 1989) and are included in SRM #1587.

While 1-NP is generally regarded as a marker for particle-bound nitro-PAH, 2-nitrofluorene (2-NF) has been advanced as a good choice for a marker of a nitro-PAH that is present in both the gas and particle phases. Advocates for the use of 2-NF in this regard point to the presence of 2-NF as one of the more common nitro-PAHs and the fact that 2-NF is a mutagen and a animal carcinogen. Accordingly, BNW has also included fluorene and 2-NF.

Several lower-molecular-weight PAHs are included because they are representative of gas-phase PAHs and are present at higher relative concentrations in DEE and RASS than the four- to six-ring compounds. Some of these lower-molecular-weight PAHs are of particular interest because an amino derivative is biologically active (2-aminonaphthalene and 4-aminobiphenyl). The corresponding nitro-compounds are present in diesel exhaust (Campbell and Lee, 1984; Paputa-Peck et al., 1983) and could be reduced either chemically or endogenously to the amino compounds or partially reduced to the nitroso or hydroxylamino derivatives. These amino compounds are also reportedly present in sidestream smoke, with the hemoglobin adduct of 4-aminopyrene being one of the most useful biomarkers of exposure discovered to date. For these reasons, BNW has included biphenyl, naphthalene, 4-nitrobiphenyl, and 2-nitronaphthalene in the list of proposed analytes.

Although BNW has cited reasoning for expanding the list of PAH and nitro-PAH analytes, method development may show that the difficulty involved in determining one or more of these analytes is too great for the purposes of the study. In those cases the particular analytes in question will be dropped after careful discussion with the Sponsor.

PAHs are well known to require care in sampling and sample processing because of their tendency to undergo oxidation or photolysis, and it is not uncommon to illuminate laboratory work areas with yellow light in an attempt to minimize photolysis. The formation of nitro-PAHs can occur when PAHs are exposed to NO_x. Thus, formation of nitro-PAH artifacts can occur during sampling, and must be minimized using short sampling times and reduced collection temperatures. BNW will carefully assess artifact formation and use the measures cited above for sampling and sample workup.

Sampling: A variety of filter types have been used to collect particulate matter to analyze RASS or DEE, including Teflon, Teflon-coated glass-fiber filters, and silica-fiber filters. Although glass-fiber filters are reported to give much higher yields for low-molecular-weight PAHs, the use of a PTFE (polytetrafluoroethylene) filter for aerosols, in conjunction with polyurethane foam or absorbents such as XAD-2 or Tenax (for capturing of more volatile compounds) is becoming a common procedure (Furton et al., 1993). In view of reports that PTFE may have less tendency to promote PAH degradation (Davis et al., 1987), BNW proposes to evaluate a PTFE filter backed with one of the common absorbents for sample collection. Although polyurethane foam is not as strong an absorbent as XAD-2, it is reported that using polyurethane foam avoids reactivity problems encountered when PAHs are collected with XAD-2 in an atmosphere containing NO_2 (Lindskog et al., 1991). Thus, the first absorbent evaluated will be polyurethane foam.

Exposure atmosphere samples will be drawn through the sampling filters for ~300 minutes each at a flow rate of ~0.5 L/minute. For RASS concentrations ranging from 1 to 10 µg/L, the expected amount of BaP collected is estimated to be 1 to 10 ng. In the case of DEE, these sampling parameters should give about 250 ng of BaP, 50 ng of 1-NP, and about 0.3 ng of 1,8- and 1,6-DNP.

Sampling will be initiated shortly after the start of exposure for each of the three concentrations of RASS test atmospheres, DEE test atmosphere, and the filtered-air exposure atmosphere. After sample collection, the filters will be stored in the dark, in sealed containers at ~5°C. The filter samples will be saved and analyzed as one set within 4 weeks after collection. Other investigators have determined that PAHs may

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be stored at room temperature in the dark for up to 118 days (Furton et al., 1993). BNW will independently evaluate sample stability.

During exposure generation testing, the collection efficiency will be assessed for BaP and 1-NP compounds by comparing breakthrough from the initial filter to the backup absorbent bed. Almost all of the BaP and 1-NP should be collected on the first filter element. Alternative collection media will be investigated if significant breakthrough of BaP and 1-NP is encountered. The gas phase compounds should exhibit minimal breakthrough to the second absorbent bed. The bed volume will be increased if significant breakthrough is observed.

Analyses will be performed to assure that degradation of the analytes does not occur on the filter/sorbent bed prior to desorption. Three sets of duplicate filters will be collected from the high concentration RASS exposure unit and the DEE exposure unit. One set of filters/sorbents will be immediately analyzed while the remaining duplicate sets will be sealed and stored at 5°C in the dark before desorption and analysis. The second set of duplicates will be analyzed after 3 weeks of storage and the third set after 6 weeks of storage.

BNW will attempt to make optimal use of the SRMs in assessing method validity, especially in assessing the recovery and extraction efficiency. The literature is replete with accounts concerning the difficulty of extracting PACs from particulate matter. Diesel particulate matter has been extensively characterized in SRM #1650 (May et al., 1992). Although this material was collected from heavy-duty diesel engines, it should serve as an excellent reference point for validating the procedures used to determine PAHs and nitro-PAHs.

Analytical Methodology. Samples will be collected using a dual element filter/absorbent. Chemical class fractionation will be performed as needed to reduce the presence of interfering substances. BNW will initially attempt to use some of recently published methods (Furton et al., 1993; Spitzer 1993), but more elaborate methods may be necessary (Lewtas et al., 1990).

As shown in Table IV-10, deuterated derivatives may be purchased for several PAHs. These compounds will be added to the extract as internal standards and analyzed by GC/MS using an HP-5971 MSD system operated in the selected ion monitoring mode with cold on-column injection onto a fused silica capillary column.

The nitro-PAHs are often analyzed using a method that can provide high selectivity for the nitro-PAHs. This approach is taken because of the low concentrations of nitro-PAHs in most samples and the concomitant need to defeat the presence of significant amounts of potentially interfering compounds. Negative-ion chemical ionization (CI) GC/MS has been widely accepted because of the general availability of the instrumentation and the high selectivity and sensitivity seen for nitro-PAHs (Schuetzle et al., 1982; White, 1985). Gas chromatography with a nitrogen-specific detector, high-resolution GC/MS, and tandem MS have also been used with good results (Paputa-Peck et al., 1983; Schuetzle et al., 1982; White, 1985).

BNW proposes the use of a TEA to analyze nitrosamines. This will also be available for use in the selective determination of nitro-PAHs. There are several accounts indicating that the TEA gives good selectivity and sensitivity for nitro-PAHs (Niles and Tan, 1989; White, 1985). Gas chromatography can be used for nitro-PAHs, but can be problematic due to degradation (generally reductions) in heated zones within the gas chromatograph and associated injection artifacts. Thus, BNW will initially attempt to separate the nitro-PAHs by HPLC/TEA.

(12) Aniline, 2-Aminonaphthalene, and 4-Aminobiphenyl. Sidestream smoke contains numerous aliphatic and aromatic amines and nitrogen-containing heterocylics. At least 30 aromatic aniline derivatives have been identified (IARC, 1985). The aromatic amines are a significant class of compounds, with 2-naphthylamine and 4-aminobiphenyl regarded as potent bladder carcinogens (Guerin et al.,

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1992). These compounds have been found in sidestream smoke at concentrations about 30 times those in mainstream smoke.

Aniline will be determined in this study as a characteristic marker of exposure to aromatic amines. However, BNW also proposes to determine 2-naphthylamine and 4-aminobiphenyl for several reasons. They are known carcinogenics and their nitro-analogs, 4-nitrobiphenyl and 2-nitronaphthalene are known to occur in diesel exhaust. Although BNW has been unable to find confirmation in the literature that biotransformation of the two nitro compounds to the corresponding amines occurs, this metabolic pathway is well known for various aromatic nitro-compounds.

The determination of 4-aminobiphenyl is of particular interest, since hemoglobin adducts of 4-aminobiphenyl have been used as a marker of exposure to tobacco smoke (Farmer, 1991) and BNW has proposed such an adduct determination as an optional study for both the DEE and RASS exposures. Measurement of 4-aminobiphenyl concentration in the exposure atmosphere is a logical complement to the adduct determination.

Analytical Methodology. The analytical methodology for determination of aromatic amines will be modeled after that described elsewhere in this proposal for determining nicotine and 3-ethenylpyridine. BNW will initially try the same extraction procedure with deuterated compounds added as internal standards, followed by GC/MS analysis. The XAD-4 sorbent tubes will be used to collect both gaseous and particle-bound amino analytes from the RASS exposure atmospheres.

As an alternative, it may be necessary to try some of the procedures described in the literature that use prefractionation (Grimer et al., 1987) and formation of pentafluoropropionamides (Patrainakos and Hoffmann, 1979). However, if plans to implement a tandem MS are realized, the use of reactant ion monitoring in conjunction with capillary column chromatography (GC/MS/MS), would very likely allow the elimination of interferences that could compromise GC/MS.

The analytical methodology will be validated by recovery studies and sample stability studies, as described previously for the determination of nicotine and 3-ethenylpyridine.

(13) Heavy Metals: A variety of metals or metal compounds are found in the particulate material from both RASS and DEE (IARC, 1985, 1989). Many of these metals, including arsenic, cadmium, cobalt, lead, chromium and nickel are associated with known adverse health effects, including cancer (IARC, 1985). Accordingly, BNW proposes that the chemical characterization of RASS and DEE exposure atmospheres include analysis of metals in particulate matter from exposure atmospheres during the subchronic and chronic studies.

Sampling. Duplicate filter samples will be collected from a nose port from each exposure unit over the duration of one 6-hour exposure day. Samples will be collected at ~0.5 L/minute on 25-mm Teflon-laminated polypropylene filters with a 0.2-μm pore size, using a calibrated flow sampler. Several filter blanks will also be collected by placing filters in the sampling device and removing the filters without drawing any sample.

Analytical Methodology. After collection, filter samples will be placed in acid-digestion bombs with 3 ml of ultrapure, concentrated nitric acid and heated at 150°C for 3 hours. After cooling, an internal standard of yttrium will be added and the contents diluted to a constant volume with deionized water. Filter extracts will be analyzed for arsenic, mercury, lead, chromium, nickel, cadmium, iron, and zinc using either ICP-AES or ICP-MS, as required to obtain the necessary sensitivity for quantitative analysis. Based on the amount of each metal determined on each filter and the volume of test atmosphere drawn from the exposure unit, BNW will calculate the concentration of each metal (micrograms/cubic meter) in the RASS and DEE exposure atmospheres.

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(14) Sulfur Dioxide. Sulfur dioxide (SO_2), a colorless gas with an irritating and pungent odor, is a common atmospheric pollutant resulting from the combustion of sulfur-bearing coals and petroleum fuels. Sulfur dioxide is present in mainstream cigarette smoke (Guerin et. al., 1992) and in DEE (IARC, 1989). At concentrations of 5 ppm or more, SO_2 has been reported to produce increased pulmonary airflow resistance in laboratory animals and humans (Greenwald, 1954).

Analytical Methodology: BNW proposes the use of a Horiba Model 350-E SO_2 monitor. This instrument measures UV fluorescence and requires none of the reagent gases or fluids associated with SO_2 analyzers based on flame photometric or coulometric detection principles. This instrument exceeds all performance specifications of the U.S. EPA and the European Environmental Testing Agency.

The lower limit of detection of the Horiba Model 350-E is 0.001 ppm SO_2. The instrument is very highly specific for SO_2; the only reported interference is water (~0.005 ppm at 100% RH). BNW will maintain the exposure atmosphere at 60 ± 10% RH, so the presence of water vapor in the system will not cause significant interference. The working range of the Horiba Model 350-E is 0 to 1.0 ppm, which BNW anticipates will be adequate.

BNW proposes to calibrate the Model 350-E SO_2 monitor on a monthly basis. Gas mixtures will be generated over the range of concentrations encountered in the test atmospheres by blending a stock standard SO_2 gas mixture with pure air using a dynamic gas calibrator. Alternatively, a certified standard SO_2 permeation tube may be used with a dynamic calibrator in which the gas stream is mixed with a metered flow of purified air to produce gas standards over the concentration range of interest. Gas standards in pressurized cylinders cannot be reliably prepared and maintained at low concentrations of SO_2).

(15) Total Hydrocarbons and Methane. Volatile organic compounds are important constituents of indoor air because they are ubiquitous and because several (e.g., formaldehyde and benzene) are suspected human carcinogens. Research to date clearly shows that cigarette smoking contributes to the volatile organic chemical burden of indoor air (Guerin et. al., 1992). Gas-phase emissions from diesel engines include C_1-C_{18} hydrocarbons, two- to four-ring PAHs, and nitrated and oxygenated derivatives of C_1-C_{12} hydrocarbons and two- to three-ring PAHs (IARC, 1989). During exposures at BNW, the atmosphere will be carefully regulated so a precise estimate of the amount of total hydrocarbons in the exposure atmospheres of DEE and RASS can be made.

Analytical Methodology: BNW proposes the use of a Horiba Model APHA 350-E ambient hydrocarbon monitor. The Model 350-E is designed for reliable, continuous, unattended measurements of total hydrocarbon, methane, and nonmethane hydrocarbon analysis, or any combination thereof. The instrument uses a flame ionization detector in combination with a catalytic hydrocarbon cutter.

The lower detectable limit of the Horiba Model 350-E is 0.02 ppm carbon with the electronic noise within 0.01 ppm carbon. The flame ionization detector is well suited for the determination of hydrocarbons and the only reported interference is ~0.2 ppm carbon by 100% RH water vapor. BNW will maintain the exposure atmosphere at 60 ± 10% RH, so the presence of water vapor in the system will not cause significant interference. The working range of the Horiba Model 350-E is 0 to 100 ppm carbon, which BNW anticipates will be adequate.

The Model 350-E ambient hydrocarbon monitor will be calibrated on a monthly basis using propane as the calibration gas. The instrument calibration will be performed using several certified standard gases encompassing the expected concentration range. A certified on-line standard of propane in nitrogen will be used to determine drift of the Model 350-E detector. This standard will be sampled before beginning a series of measurements and routinely during the measurements to determine detector drift within a typical exposure day.

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b. Aerosol

(1) Tapered Element Oscillating Microbalance (TEOM) Mass Monitor. On-line monitoring of the mass concentration of RASS and DEE in the exposure units will be accomplished by a TEOM monitor (Model 1400).

The TEOM is a gravimetric instrument capable of measuring the concentration of particles in the range of 50 $\mu g/m^3$ to several grams/cubic meter. The system draws sample air through a filter at a constant, calibrated rate. A Teflon-coated borosilicate glass filter is mounted on top of an oscillating tapered sampling tube. An electronic feedback circuit maintains the oscillation of the tube at its natural frequency. This frequency is directly dependent on the mass collected on the filter. By accurately measuring the oscillation frequency, the mass can be determined. The concentration is then calculated from the measured mass collected on the filter and the measured sample volume through the filter.

TEOM readings will be primarily used to control RASS and DEE TPM concentrations and to report temporal concentration stability during the 6-hour exposure period. The TPM determined from Cambridge-filter samples will also be reported as the average 6-hour exposure concentration.

Because the mass concentration of the DEE is continually changing as the engine is cycled, a single TEOM will be required to monitor the DEE exposure unit pair. This TEOM will report the average DEE mass concentration during the 23-minute engine cycle. During the subchronic study, the DEE TEOM will be valved to alternately monitor the 10- and the 3-mg/m^3 exposure units for individual 23-minute cycles. A second TEOM will be multiplexed to monitor the mass concentration of RASS in each of the RASS exposure unit pairs. This TEOM will also monitor the mass concentration in the filtered-air exposure unit pair. The output of the TEOM monitors will be interfaced to the main data acquisition and control system by a dedicated computer running proprietary software developed by BNW.

The TEOM system will be calibrated by attaching a filter of known mass on the tapered element. The corresponding frequency will be measured and the "spring constant" of the system will be calculated. From this constant, the mass can be calculated at other frequencies of oscillation. This calibration will be checked against separate gravimetric filter samples taken from an exposure port at the same time the TEOM sample is taken.

(2) Realtime Aerosol Monitor (RAM). On-line monitoring of the RASS smoke as it enters the distribution line from the aging room will be accomplished with a dedicated RAM. The RAM readings will be used only to monitor and control the stability of the TPM concentration of RASS in the aging room.

The RAM uses a pulsed light-emitting diode in combination with a silicon detector to sense light scattered over a forward angle of 45 to 95° by the particles traversing the sensing volume. The instrument will respond to particles in the 0.1- to 20-μm diameter size range. Although the geometric diameter of cigarette smoke approaches the minimum of this range, the concentration in the aging room will be high enough to assure a RAM response sufficient to determine the stability of the concentration for control purposes.

The output of the RAM will be automatically read and recorded by the data acquisition and control system. As each voltage is read, it will be compared to limit values. If limit values are exceeded, the system will sound an alarm to indicate that the generation system must be adjusted.

An approximate calibration of the RAM will be performed by correlating instrument response (voltage) with TPM values from weekly filter samples collected from the aging room at the point RASS enters the distribution line.

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(3) Total Particulate Matter (TPM) Filters. Aerosol mass measurements of both RASS and DEE will be performed by taking duplicate Cambridge-filter samples from two exposure ports from each exposure unit. The filters will be analyzed gravimetrically. The filter samples will be taken during the entire 6-hour exposure period each day. The TPM determined from these samples will be the reported average exposure concentration for the exposure period. The UV-PM method may also be used to determine TPM for samples with insufficient TPM for gravimetric determination.

(4) Aerosol Particle Size Distribution. Ingebrethsen and Sears (1985) evaluated the particle size distribution of ETS smoke at different mass concentrations (Table IV-11).

TABLE IV-11. Aerosol Properties of Environmental Tobacco Smoke[a]

Particle Mass Concentration ($\mu g/m^3$)	1.4	28	226
Average diameter (μm)	0.098	0.123	0.141
Mass median diameter (μm)	0.185	0.198	0.21
Particle number <0.1 μm (%)	73	54	39
Particle mass <0.1 μm (%)	17	8.5	5.5

[a]Dilute sidestream smoke aged for 1 hour in an 18-m^3 chamber

These results suggest that the particle size of ETS increases with increasing mass concentration. The mass concentration of the ETS (RASS) smoke to be generated in our experiments will initially be ~20 $\mu g/L$ in the aging room. It is expected that the particle size will be somewhat larger than above, but probably less than 1 μm in average diameter.

Particle size distribution of the RASS aerosol will be determined in the aging room and in the exposure units before beginning the study. In the prestart phase, applicability of various techniques will be evaluated, including:

- An optical particle counter (Model HSLAS-065, Particle Measuring Systems Inc.; Boulder, CO)
- An electrical mobility analyzer with a condensation nucleus counter (Model DMPS; TSI, Inc.; St. Paul, MN)
- A Mercer-style cascade impactor (In-Tox Products, Albuquerque, NM).

The Model HSLAS-065 is a highly sensitive laser aerosol spectrometer that relates the light scattered by a single particle to its size. It operates within a size range of 0.065 to 1.0 μm in diameter.

Electrical mobility analyzers create a known charge distribution in an aerosol. By measuring the electrical mobility of particles in a well-defined electrical field, the particle size distribution can be calculated. The size range of the Model DMPS is 0.02 to 1.0 μm in diameter, with decreasing efficiency as the particle size approaches 1.0 μm.

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Mercer-style cascade impactors separate aerosol particles into eight size ranges as the sample is drawn through a series of stages with jets and the particles impact on the glass collection plates. The effective cut-off diameters of the stages vary from about 0.2 to 4 μm. The amount of material collected on each impaction stage will be determined by UV/PM chemical analysis.

A wide size range will be covered with these three techniques, which will enable us to choose the method most applicable to the aerosol system. It is estimated that most of the particles in the aging room and the exposure atmosphere will be between 0.1 to 1.0 μm in size. Assuming that the performance of the Model HSLAS-065 with RASS is verified during the prestart effort, BNW proposes to use this optical particle counter routinely during the subchronic and chronic studies to monitor particle size distribution in the aging room and exposure units.

The method for monitoring the particle size distribution of DEE will be selected during the prestart phase in a manner similar to the RASS study. DEE is expected to have a somewhat larger particle size distribution. It is anticipated that a combination of the optical particle counter and the cascade impactor will be used for routine monitoring.

Combustion aerosols such as cigarette smoke and diesel engine exhaust are very complex systems with thousands of components. Their aerosol properties are time-dependent, with continuous transfer of material between particulate and vapor phases, as well as between particles of different sizes. Highly concentrated aerosol systems undergo coagulation, which can reduce the number of particles and increase the average particle diameter. Volatile components can be lost from the particulate phase upon dilution. It is also expected that the chemistry of the combustion particles will vary somewhat with particle size.

In the light of these complexities, when size-distribution data are reported, the dilution level, the age of the smoke, and details and shortcomings of the measurement technique should be clearly stated. During the prestart phase, we intend to evaluate the techniques mentioned above and choose the most applicable and repeatable methods for the routine analysis of particle size distribution in our exposure chambers. It is likely that the particle size data will not be absolute or definitive, but it should provide a means to evaluate the consistency of the aerosol particle size distribution throughout the studies.

c. Environmental

(1) Temperature. Temperatures will be measured using T-type thermocouples connected to the data acquisition and control system. Temperatures from the aging room, at a single port of each exposure unit, in each exposure unit cabinet, and from the animal housing room will be automatically recorded. Calibration of this system will be accomplished by comparison with a certified mercury thermometer and a temperature-controlled water bath.

(2) Relative Humidity (RH). The RH will be calculated from the temperature measured by a thermocouple and the dew point measured by an EG&G Model 911 DewAll digital humidity analyzer (EG&G, Burlington, MA). A single hygrometer will be multiplexed to the various measurement locations with a computer-controlled valve system. RH from the aging room, at a single port of each exposure unit, and from all animal housing rooms will be automatically recorded. The humidity analyzer will be calibrated by comparison to a certified, portable, EG&G Model 911 dew-point hygrometer.

(3) Air Flow. System air flows will be determined by measuring the pressure drop across calibrated orifices or venturis using a single Validyne Model CD-12 carrier demodulator transducer (Validyne, Northridge, CA) interfaced to a Validyne Model MP-45 pressure transducer. This system will be calibrated by a Dwyer Mark II (Dwyer, Michigan City, IN) water manometer. Exposure unit inlet and exhaust flows, as well as distribution line flows, will be measured by a single-pressure transducer multiplexed to the various measurement locations with a computer-controlled valve system. Flow will be automatically

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recorded at each location. The flow system will be calibrated by comparison to a certified flow calibration unit.

(4) Sound Level. Sound levels will be measured in both the animal exposure rooms and the DEE room. Measurements in the engine room will be made to determine if ear protection will be required for workers in the room. Measurements in the animal rooms will be made to assure that the exposure equipment does not generate sound levels that could significantly contribute to animal stress. Sound levels will be measured during a period of normal activity with all exposure generation equipment operating. Both A-weighted and flat-frequency measurements will be taken, the latter being more appropriate for interpreting noise levels heard by rodents. The former, which more closely approximates the sensitivity of the human auditory system, will be included as a reference for interpreting noise levels sensed by humans.

The measurements will be made with a precision integrating sound level meter (Model 1988, General Radio, Concord, MA) with a ½-inch random incidence microphone. The frequency response of this meter is 5 Hz to 20 kHz. This instrument meets the requirements of ANSI Standard S1.4-1971 for Type 1 sound level meters and ANSI Standard S1.11-1966 for octave band filter sets. The meter will be calibrated on the day of the measurement using a General Radio 1986 Omnical sound level calibrator at 1000 Hz.

2. Prestart Validation

a. Temporal Stability

(1) Total Particulate Matter (TPM). Although the TEOM will not be used to report average concentration for the 6-hour exposure period (gravimetric or UV analysis of filter samples will be used for this purpose), its response will be used to indicate the relative concentration during the course of an exposure and will be used as feedback for manual control of the exposure concentrations. A single TEOM will be dedicated to monitoring the DEE exposure unit pair while a second TEOM will be multiplexed to monitor the RASS and filtered-air exposure unit pairs. The TEOM aerosol concentration measurements will be made in one of each RASS and DEE exposure unit pair twice/hour. These measurements will be used to demonstrate and control the temporal stability of the aerosol mass concentration during the exposure period. The average TPM concentrations will be controlled to within ±20% of the target value. The variation in daily concentration will be controlled to within ±20% relative standard deviation (RSD) from the mean. Prior to the start of each study, concentration stability within the desired target range will be demonstrated during three successive exposure days.

Before the start of each study, the concentration buildup and decay times in the aging room and the RASS exposure units will be determined from TEOM measurements. For these measurements, the TEOM will sample from the RASS distribution tube as it exits the aging room or from each single exposure unit approximately once every 2 minutes. The time for the concentration to reach 90% of its target value at the start of the exposure (T_{90}) and the time for it to decay to 10% of the target value at the end of the exposure (T_{10}) will be reported. The RASS generation system will be operated for sufficient time to achieve stable concentration in the aging room before the start of animal exposures. Due to the cycling nature of the DEE generation system, no buildup and decay times will be determined for the DEE exposure units; however, the DEE generation system will be operated for one engine cycle prior to the start of animal exposure. Both RASS and DEE animals will be exposed for a period of 6 hours plus T_{90} (T_{90} will be determined only for RASS and will be used for the entire study).

The relative aerosol mass concentration in the RASS aging room will be monitored by a RAM dedicated to this purpose. RAM readings will be recorded at ~15-minute intervals during the concentration buildup period and during the 6-hour exposure period.

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(2) Carbon Monoxide (CO). Carbon monoxide will not be used as a concentration control parameter; however, CO concentration, in combination with aerosol concentration as determined by the TEOM, can be an excellent tool for recognizing and diagnosing possible problems with the test article generation and distribution systems. CO concentration measurements will be made in one of each RASS and DEE exposure unit pair twice per hour during exposure. If the generation and delivery systems are functioning properly, variations in CO concentration should be directly proportional to variations in aerosol concentration.

During prestart validation, the normal relationship between CO concentration and aerosol concentration will be determined.

(3) Aerosol Particle Size. To assure that the particle size distribution is not affected by the generation and distribution system during the course of an exposure, particle size analysis will be performed from a single port from the high-concentration DEE and RASS exposure units during the first and last hour of a typical exposure period. The diesel engine will be run at a constant speed and load during this test to eliminate the temporal variation in DEE concentration.

b. Spatial Uniformity

(1) Total Particulate Matter (TPM) and Carbon Monoxide (CO). The spatial uniformity of the aerosol and CO concentrations within the exposure unit will be determined by measuring the aerosol mass using the TEOM and the CO using the CO monitor. Concentrations will be measured at one exposure port on each level of each exposure unit. The diesel engine will be run at a constant speed and load during this test to eliminate the temporal variation in DEE concentration.

(2) Aerosol Particle Size. Prior to the start of each study, particle size measurements will be made at a single exposure port at the top, middle and bottom of each exposure unit during routine generation to establish the particle size distribution of the aerosol and to demonstrate spatial uniformity.

c. Exposure Atmosphere Characterization. The exposure atmosphere for RASS and DEE will be characterized by performing the chemical-specific measurements cited in Table IV-4. The validation of these methods and associated measurements will be summarized in the prestart report. Finalized SOPs for each of the cited measurements will also be included in the prestart report.

Particle Size. Particle size distribution measurements will be repeated at one port per exposure unit once during the subchronic study and at least once per month during the chronic study.

D. EXPOSURE DATA ACQUISITION AND CONTROL

1. System Description

The main data acquisition and control system for the studies will consist of an Apple Macintosh computer interfaced with an HP Model 3497A data acquisition control unit (Hewlett-Packard, Hollywood, CA). Input from analog instruments and thermocouples will be interfaced using HP analog input cards. HP digital input cards will be used to monitor alarm conditions and on/off status devices. HP digital actuator cards will operate control relays.

System software has been developed using LabVIEW (National Instruments, Austin, TX) data acquisition, analysis, and presentation software. This programming application uses the computer display for both a control interface and data display. A mouse is used to "point and click" on virtual control elements such as "buttons" and "switches" that appear on the display screen. The software allows each exposure group to be exposed independently of the other groups, allowing staggered start times.

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Specific information relating to the data acquisition program (such as instrument assignments, measurement frequencies, calibration parameters, alarm limits, and alarm reactions) will be entered into spreadsheet-like display windows on the computer screen. To assure GLP compliance, any changes in these values will result in a printout of the old window, the new window, the date, operator's name, and a comment field for explaining the change. Changes will be highlighted in the new window.

Alarm settings include noncritical and critical, upper and lower alarm limits for each data type at each measurement location. The response to particular alarm situations is defined by data alarm response types that can be assigned to any location for each of the four possible alarm conditions. Each alarm response is a self-contained subprogram that, when invoked, will take the appropriate action at the location from which it was called. All data that exceed alarm limits will be printed and stored.

Data will be stored to disk and printed to paper as they are collected, thus assuring minimal data loss in the event of a system failure. Due to the relative durability of paper, the printed record will be officially considered the raw data. The following information will be printed for each measurement: time of acquisition, raw data value, calculated data value, units of measurement, identification of the type of data, the measurement location, and any indication of alarm limits exceeded. At the end of each exposure day, all data recorded to disk will be copied to a central file server over a local area network. These files will be write-protected (i.e., the data cannot be changed in these files). The files on the file server will be routinely backed up on streaming tape. Data editing and analysis will be performed only on backup editing files by qualified and authorized personnel.

Table IV-12 lists the parameters that will be recorded by the data acquisition system, as well as the monitoring locations and recording frequency. The on-line monitoring devices are described in Section IV.C.1.

2. Data Analysis

Exposure data analyses and summations will be performed using spreadsheets and macro routines (Excel, Microsoft Corporation, Redmond, WA) developed at BNW.

3. System Documentation and Validation

System description documentation and SOPs will be written to cover description, operation, calibration, validation, and verification of all exposure systems; data acquisition and control systems and software; and data analysis software. Documentation and validation will satisfy the U.S. EPA GLP Toxic Substance Control Act regulations, 40 CFR 792.

E. INDUSTRIAL HYGIENE AND SAFETY

Throughout the course of the studies, BNW will protect the health and safety of personnel involved and will comply with pertinent environmental protection requirements. Health and safety planning begins with the design of the logistics and the physical layout, with emphasis on the use of engineering controls to contain diesel fuel vapors, diesel exhaust, cigarette smoke, and titanium dioxide, and prevent their escape into the work space. The effectiveness of engineering controls will be ascertained by room air monitoring using continuous, real-time instruments. Concentration readings in excess of the action level (one-half the TLV) will trigger an audible alarm and immediate shutdown of the generation system. Sampling will also be performed initially to monitor the presence of diesel fuel vapors in the room air to ensure that no fuel leaks into the work space. Room air monitoring will be performed throughout the studies in Rooms 511, 519, 523, and 531.

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TABLE IV-12. On-Line Monitoring Parameters

Parameter	Monitor	Location	Number of Locations	Recording Frequency (per Hour)
Temperature	Thermocouple	Exposure Unit	10	once
		Exposure Unit Cabinet	10	once
		Exposure Room	2	once
		Animal Room	1	once
		RASS Generator Room	1	once
		RASS Aging Room	1	once
Relative Humidity	Hygrometer	Exposure Unit	10	once
		Animal Room	1	once
		RASS Generator Room	1	once
		RASS Aging Room	1	once
Air Flow	Orifice Meter	Exposure Unit Inlet	10	once
		Exposure Unit Exhaust	10	once
	Venturi	RASS Aging Room Flow	1	once
		DEE Distribution Line Flow	1	once
TPM	TEOM "A"	RASS Exposure Unit Pair	3	twice
	TEOM "A"	FA Exposure Unit Pair	1	twice
	TEOM "B"	DEE Exposure Unit Pair	1	twice
	RAM	RASS Aging Room	1	twice
CO	CO "A"	RASS Exposure Unit Pair	3	twice
	CO "A"	FA Exposure Unit Pair	1	twice
	CO "A"	Generator & Exposure Rooms	5	twice
	CO "B"	DEE Exposure Unit Pair	1	twice

KEY: CO = Carbon monoxide; DEE = Diesel engine exhaust; FA = filtered-air; RAM = Realtime aerosol monitor; RASS = Room-aged sidestream cigarette smoke; TEOM = tapered element oscillating microbalance mass monitor; TPM = Total particulate matter.

Carbon monoxide is a chemical compound common in both diesel exhaust and cigarette smoke. Personnel exposure sampling will involve breathing-zone sampling of CO, in lieu of sampling a wide variety of chemicals that may be present in the two test materials. Results of CO sampling will be used as an index to estimate personnel exposure to other major chemical components in diesel exhaust and cigarette smoke, e.g., aldehydes, NO_x, CO_2, and hydrocarbons. Correlation of CO to these other major chemical components will be done during prestart activities. No attempts will be made to distinguish the contribution of diesel exhaust generated CO exposure from that of cigarette smoke.

The extent of personnel breathing-zone exposure to CO will be initially characterized based on the exposure assessment strategy developed by the American Industrial Hygiene Association (1991). Once exposure characterization is complete, subsequent sampling will be performed periodically for surveillance or under special circumstances. Breathing-zone sampling results may be supplemented by means of

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monitoring CO concentrations in the exhaled air at the end of work shift as recommended by the American Conference of Governmental Industrial Hygienists (1992). Personnel breathing-zone exposure to carbon monoxide will be monitored in Rooms 511 and 519 and occasionally in Rooms 523 and 531.

In addition to potential exposure to test chemicals, excessive noise levels may also present a hazard to personnel in the diesel engine room and therefore will be evaluated. If a noise hazard does exist [>85 dB(A)], applicable Occupational Safety and Health Administration (OSHA) hearing conservation program requirements will be followed (29 CFR 1910.95). Personnel will be provided appropriate hearing protection and caution signs will be posted at the entrance(s) to the rooms. Since personnel entering the diesel engine room stay for only brief periods to start, stop, or service the engine, noise exposure will be minimal. Nevertheless, appropriate hearing protection will be required and room access will be restricted.

Where engineering controls may not always be completely effective, the protection of personnel may be supplemented by establishing certain safe work procedures supported by training and using personal protective equipment. Personnel involved in this study will be provided training to comply with the OSHA requirements for hazard communication (29 CFR 1910.1200 and 1450). A health and safety protocol will be developed and posted in the study area. This protocol will describe available data on health and safety effects from exposure to the test materials, level of personal protection needed, waste disposal requirements, spill control and emergency procedures.

Related health and safety considerations will include fire protection, proper air balance in the work spaces, and prevention of environmental releases.

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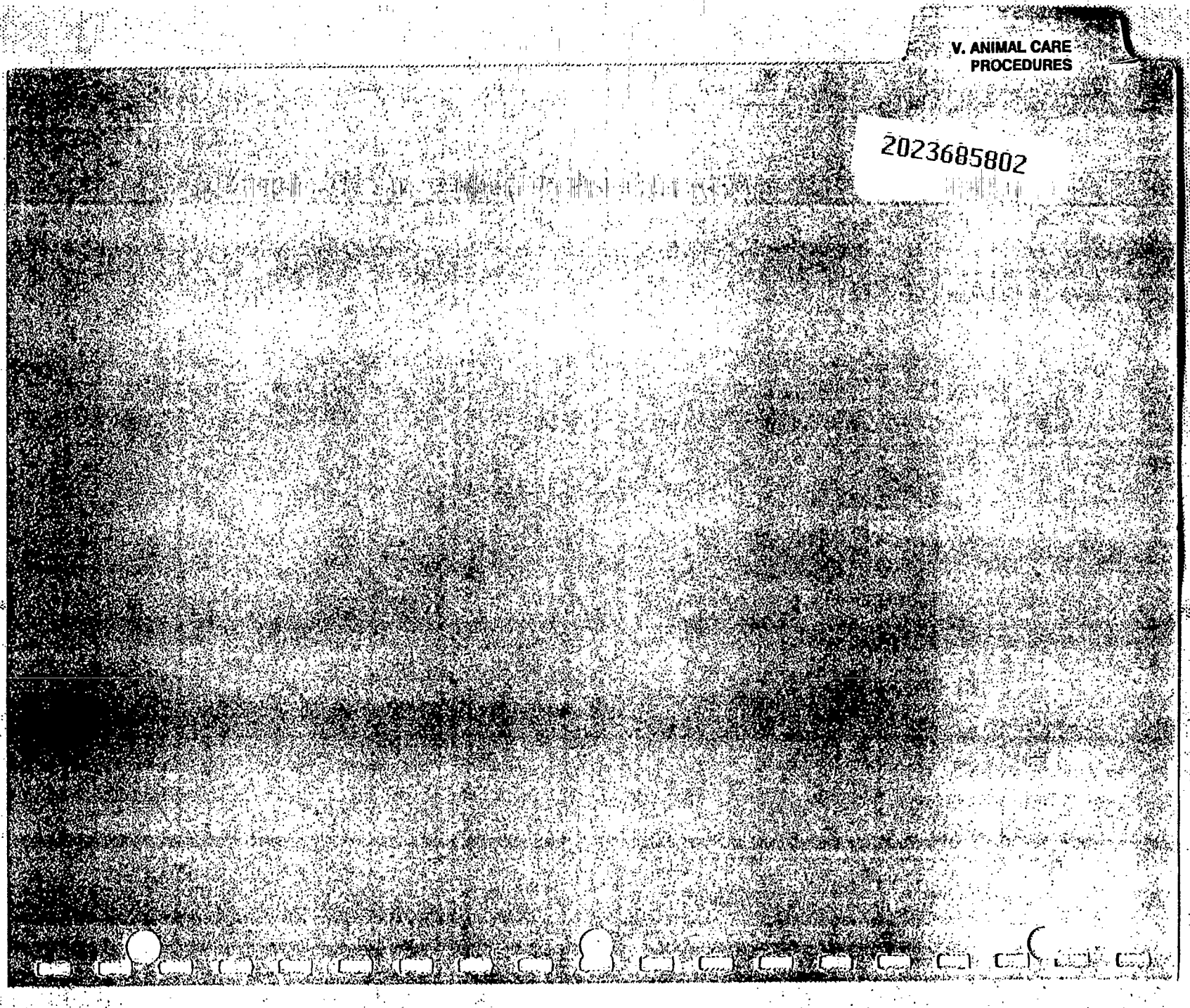

V. ANIMAL CARE PROCEDURES

A. ANIMAL FACILITIES, PATHOGEN CONTROL PROCEDURES, AND ANIMAL CARE PERSONNEL

1. Animal Facilities and Equipment

LSL-II is designed primarily for large life span inhalation studies in rodents. The laboratory provides spacious animal rooms in a two-corridor (regulated/clean) layout. Traffic into animal housing and exposure areas is strictly controlled with access only from the clean corridor. Monitoring and other study support functions are conducted remotely when appropriate in order to reduce equipment and personnel traffic in the animal areas. Total animal space in LSL-II is 15,500 ft^2. An additional 5,700 ft^2 of animal care support space is used for the cage washing facilities, change rooms, offices, storage areas, and exposure suite control rooms (Figure IV-1).

Major equipment (heating, cooling, and ventilation) is provided in duplicate or with other back-up. The building contains a large diesel-powered generator that can maintain normal temperatures and good ventilation in the animal facility if normal electrical power is lost. All essential systems are on a routine monitoring and preventative maintenance schedule.

The ventilation system provides approximately 18 air changes/hour to animal rooms in the facility. Air pressure differentials within the facility are such that the clean corridor remains positive to the animal rooms, which are positive relative to the regulated corridor. Some rooms are maintained negative to both corridors for safety reasons (containment of test article aerosols). Street areas (outside the barrier) are at the lowest pressure.

All animal room doors remain closed except for passage or for special purposes. While closed, the room is an isolated unit with virtually no airflow in or out except through that room's intake and exhaust ducts. An absolute (i.e., HEPA) filter is located in each room's intake duct to remove airborne particulate contaminants. To ensure entrapment of any potentially noxious particulate release within a given room, exhaust air passes through two successive HEPA filters before being exhausted from the building. Single HEPA filters are located in the exhaust ducts of each room; the second filtration occurs just before the air leaves the building.

Room temperatures are individually adjustable and are automatically recorded and monitored in a central location. Room temperatures generally fluctuate no more than ±2°F. Temperatures are recorded at 60-minute intervals on a remote chart recorder and an alarm system makes immediate contact with responsible personnel on a 24-hour basis should temperatures be less than 72°F or greater than 78°F. Lights, controlled by automatic timers, are routinely set on a 12-hour light/12-hour dark cycle.

The cage washing area is divided into a clean and dirty side. Positioned in the wall separating the two sides are a large pass-through cage and rack washer, a smaller cage and bottle washer, and a pass-through autoclave. A second autoclave located in the clean wash area will accommodate large racks with cages or bulk supplies. Both autoclaves operate to 270°F, have digital timers and a three-pulse vacuum.

All animal watering in the facility, except for special purposes, is by means of automatic water systems attached to the cage racks. Nearly all racks and cages used in the facility, including attached automatic watering systems, are autoclavable.

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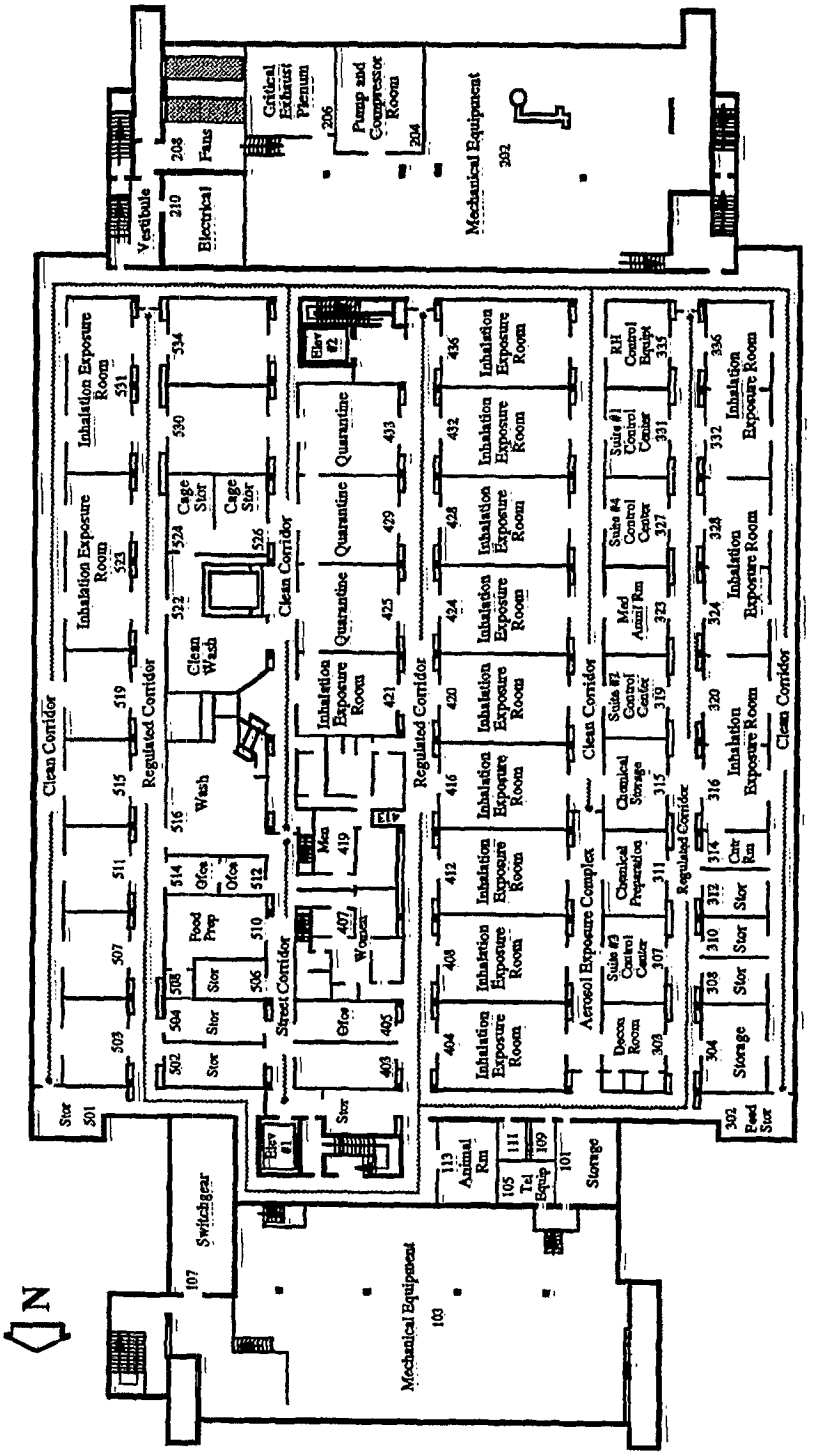

FIGURE V-1. Life Sciences Laboratory-II Basement Floor Plan

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Animal drinking water is provided by the city of Richland's system. Water quality is monitored, as required by state and federal regulations, by the city of Richland. In addition, LSL-II animal drinking water is analyzed for contaminants at least once each year. We do not acidify or chlorinate animal drinking water; however, the water is chemically softened and filtered to remove excess minerals. The sodium ion concentration in the softened water is approximately 50 ppm.

2. Animal Care Procedures

The operation of the LSL-II animal facility is focused on maintaining animals in a clean, stable environment and maintaining them free of infectious diseases. Sanitation and barrier procedures are well-defined and strict adherence to these procedures by all personnel is enforced throughout the facility.

Barrier procedures for the facility provide two levels of control. The first level is designed to prevent introduction of pathogenic microorganisms into the facility. If this should fail at any time, the second level provides good protection against movement of the pathogen from one room to another within the facility.

a. Preventing Introduction of Pathogens into the Facility

(1) Animal Control. The first level of control begins with careful selection of sources of pathogen-free stock and limiting those sources to reduce the risks of introducing pathogens from a supplier. Limiting the sources also generally provides better control over transportation arrangements and thus potential exposure to pathogens in transit. Animals arrive at the nearby Pasco airport and are promptly picked up in one of two climate-controlled BNW animal transport vehicles.

Our health-screening procedures have been reliable for detecting the presence of pathogens in incoming animals. Bacterial culture, serologic testing, and microscopic examination of selected tissues are used to detect pathogens and lesions in the animals examined. Our usual procedure is to promptly kill all animals in the group if a pathogen is found that is judged significant with respect to its possible effect on the study for which the animals are to be used or on other ongoing studies in the facility.

(2) Control of Personnel, Equipment, and Supplies. Our barrier procedures strictly control movement of personnel, supplies, and equipment into and within the facility. Personnel entry is limited to a "need-to-go" basis. Equipment must be thoroughly sanitized before entry and once in, generally remains within the barrier area. Supplies are removed from outer shipping packaging and sterilized, if appropriate, before passing into barrier areas.

b. Preventing Pathogens from Moving from One Room to Another in the Facility

(1) Personnel Control. The LSL-II animal facility is operated to allow for some difference in the extent of barrier protection for individual rooms. However, all animal rooms, including quarantine rooms and animal exposure rooms are operated according to our clean room regulations. Access into these rooms is only from the clean corridor. Personnel are required to wash hands and arms (or shower) and change into sterilized clothing before entering the clean corridor. Exit from animal rooms and animal exposure rooms is permitted only into the regulated corridor. Once out of the room, re-entry is permitted only by way of the clean corridor after a clothing change and hand and arm wash in the change room. When exiting a quarantine room, personnel must return directly to the change room, remove their clothing and wash their hands and arms before proceeding to any other area.

(2) Animal Control. Animals are not permitted in the clean corridor. Therefore, animals are moved from the quarantine room to the exposure room in the regulated corridor. The transfer is made in closed 2.3-m^3 whole-body exposure chambers. The transfer chambers are moved through the regulated corridor to the side of exposure room doors, then chamber doors are opened and the cage units are transferred through the exposure room doors to a clean rack in the exposure room or adjacent animal

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room, if housing is not in the exposure room. For the studies proposed, the animals are expected to be quarantined in the same rooms in which they will be held after exposures start.

All operations with rodents in the facility are designed to avoid the necessity of moving animals through the corridor for exposure or other experimental procedures and then returning them to the animal housing room. For these studies, this will be accommodated by providing a door in the corridor to create a suite between adjacent rooms that are to be used for housing and exposure.

Animals from ongoing studies within the facility are monitored for pathogens using serologic, culture, and direct examination methods. Besides providing data for individual studies, this information is used for pathogen control within the facility.

(3) Control of Equipment and Supplies. Equipment is washed in the cage washer and sterilized or sanitized as thoroughly as possible before being moved through the clean corridor into an animal room or animal exposure room. Soiled animal racks and cages are moved through the regulated corridor to the wash area. When practical, other equipment is dedicated to a room and remains there as long as it is needed. Sanitation procedures for such equipment are carried out in the room. If it must leave the room, exit is via the regulated corridor.

c. General Sanitation Procedures. Floors in all animal rooms, animal exposure rooms, and corridors are sanitized daily. Animal room and exposure room floors are hosed off once a day with NPD UNICIDE 128 (a sanitizer containing quaternary ammonium compounds) solution in 100 ± 10°F water. Corridor floors are washed once per day using the NPD UNICIDE 128 solution in a mechanical floor scrubber. More thorough scrubbing of floors and walls in rooms and corridors is done on an as-needed basis.

All cages and cage racks are washed every 7 days when in use. Cleaning agents dispensed during various washing cycles of the cage and rack washer (Better Built, Model 6300 SP) are Uri-Solv, pH Control, and PRL-18. These are all products of Pharmacol Research Laboratories, Inc. The initial cycle is with Uri-Solv, a phosphoric acid solution used to remove mineral deposits. After the first cycle, pH Control, a sodium hydroxide solution, is added to neutralize the acid. PRL-18, a chlorinated alkaline cleaner, is added to the second (last) wash cycle. This cycle is followed by two thorough rinse cycles using fresh, clean, ~180°F water. After washing, animal equipment is held in a clean storage area. When needed, it is moved through the clean corridor to the exposure room or animal room. All excreta pans beneath the mouse cage units are removed every morning and replaced with clean pans that have been washed in the cage washer.

The LSL-II animal facility is maintained free of wild rodents and other vermin by facility design and construction features, and by controls on the movement of equipment and supplies into the animal facility. No pesticides are used in the facility.

3. Animal Care and Use Committee and Accreditation by the American Association for the Accreditation of Laboratory Animal Care (AAALAC)

BNW has an Animal Care and Use Committee (Dr. Rowe, Chairman) with representation from each organizational group within BNW that uses animals. The Legal Department is also represented on the Committee. The Committee's non-BNW affiliated member is a biologist-educator from a local elementary school. Initiation of all studies involving the use of animals is dependent on the Animal Care Committee's review and approval. Committee functions have been defined in the Animal Welfare Assurance Statement submitted to the Office for Protection from Research Risks, National Institutes of Health (NIH). Our PHS (NIH) assurance number is A-3353-01; our USDA registration number is 91-R-006.

BNW's Animal Care Program (and facilities) has had continuous full accreditation by AAALAC since 1976. The next AAALAC site visit will be in 1994.

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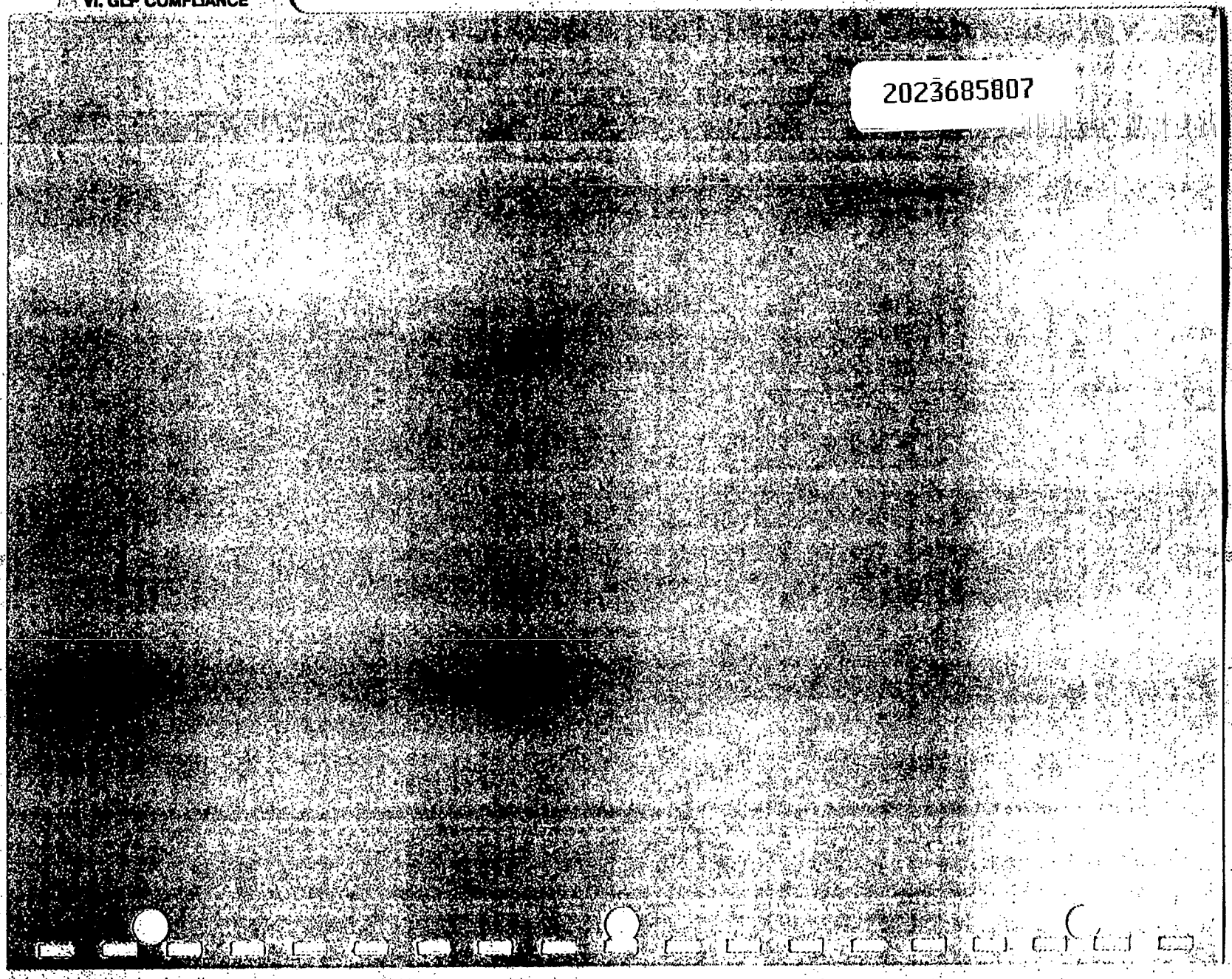

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VII GOOD LABORATORY PRACTICES COMPLIANCE

Except where deviations are specified by the contract, these studies will be conducted in compliance with the EPA Good Laboratory Practices (GLP) regulations, 40 CFR 792.

A. GOOD LABORATORY PRACTICES (GLP) IN THE TOXICOLOGY DEPARTMENT

BNW has a QA Unit that resides in the Life Sciences Center. It is staffed by two full-time auditors and two clerks who report directly to the Manager of Operations in the Life Sciences Center. The QA Unit staff is responsible for providing independent verification to management that the testing facility is in compliance with relevant regulations. In fulfilling this responsibility, the QA Unit monitors compliance with those regulations and BNW SOPs through surveillances, audits, and data reviews. Written reports of compliance, which note any problems and corrective actions taken (§792.35), are then submitted to Task Leaders involved, Line Management in the Toxicology Department, and the Manager of Operations.

B. GOOD LABORATORY PRACTICES FOR THE PROPOSED PROJECT

1. Personnel

The Principal Investigator (PI) has the requisite education, training and experience to conduct the proposed studies. He represents the single point of control and has the overall responsibility for the technical conduct of the studies. The Study Director is responsible for the day-to-day decisions that involve the study, as specified in §792.33. All Task Leaders responsible for specific tasks have the education and experience necessary for their assigned functions. Technicians have, or if necessary will receive, training in the specific areas in which they will be working.

2. Facilities

In compliance with 40 CFR 792, BNW management will assign adequate facilities suitable for the proposed work. Animal facilities are operated in compliance with all applicable regulations.

3. Quality Assurance Unit

a. Surveillances and Audits. The QA Unit staff will monitor study activities through routine surveillances and data audits in compliance with the GLP regulations [§792.35(b)]. Study activities that will be reviewed during surveillances include: animal receipt, quarantine, health screen and release to study, randomization, and identification; exposure; calibration of the exposure system; body weight measurement; clinical observations; computer data collection; clinical pathology; special studies (ophthalmologic exams, minute volumes, EKGs, blood pressure measurements, etc.); necropsy; tissue trimming; and slide preparation. During the surveillance activities, conformance to study protocol, SOPs, and other applicable standards is assessed. The methods used in conducting surveillances are detailed in the SOP, *Inspection of Study Activities*.

The study protocol will be reviewed and signed by the QA Auditor. In compliance with §792.35(b), study data will be audited quarterly or, as in the case of one-time events, when generated. Data to be reviewed include: protocol amendments and deviations; exposure; body weights; clinical observations, clinical pathology, special studies, system calibration, mortality, necropsy, and histology; pathology, wet tissues, and slide/block match-up. Each set of data will be reviewed for compliance with the study protocol, SOPs, and applicable Sponsor-generated Statement of Work requirements. The Development Phase and Prestart Report, and the Final Study Report will be validated with the raw data. Audit procedures and report validation are described in the SOPs *Protocol Amendments*, *Data and Report Audits*; *Pathology Data Audits*, and *Exposure Data Audit*.

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b. Auditor Training. BNW selects staff with technical experience for the QA Unit. The present members of the QA Unit staff have laboratory experience ranging from 13 to 20 years. This knowledge is augmented with a continuing education program of internal and external training, seminars and classes. The QA Unit staff participate in the in-house training provided to the technical staff. In addition, they attend meetings in which the study activities and problems are discussed so that they can be better informed about the activities and data they are reviewing.

The staff of the QA Unit have attended various local courses of QA given by Columbia Basin Community College and the Quality Training and Resource Center (a multicontractor organization designed to provide comprehensive training in QA, Safety, and Environmental Compliance). Each year BNW provides funds for the auditors to attend annual meetings and associated training seminars of the Society of Quality Assurance. Ms. Gelman is an American Society of Quality Control (ASQC)-certified Quality Auditor.

c. Quality Assurance Procedures. The QA Unit has SOPs that address surveillances, auditing activities, record maintenance, and record control. These SOPs are reviewed annually and updated as needed. The process of SOP maintenance is described in the next section.

4. Standard Operating Procedures

BNW has a defined SOP process, which is described in SOP, *Production and Use of Standard Operating Procedures*. Procedures for study activities are prepared by the professional staff and approved by BNW Line Management prior to the start of the study. They are reviewed annually and again when established procedures are modified. The QA Unit retains a current copy of all SOPs and maintains the historical file of retired SOPs.

Official copies of new or revised SOPs are issued to the workplace and the Task Leader by the QA Unit. An unofficial copy, with a signature sheet, is routed for review to all staff who will be using the SOP. Group training sessions are routinely held to discuss the particulars of SOPs (including any changes), then individual "hands on" training is provided by the Task Leader (or designee), as needed, to assure the staff is well-versed and proficient in conducting the procedure. Any deviations to SOPs during the course of the study must be authorized by the PI (or his designee).

Conformance to SOPs is monitored during QA Unit surveillances and audits. Deviations from standard procedures are discussed with the PI and described in the QA Unit report to Line Management.

5. FDA Inspections

The most recent FDA inspection at BNW was conducted August 1992. The laboratory was in compliance with 21 CFR 58.

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VII. CAPABILITY & MGMT PLAN

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VII. ORGANIZATION, EXPERIENCE, CAPABILITY AND MANAGEMENT PLAN

A. BATTELLE, PACIFIC NORTHWEST LABORATORIES (BNW)

Battelle Memorial Institute was created in 1923 by the will of Gordon Battelle, an Ohio industrialist. Since then it has become one of the world's largest independent, not-for-profit research organizations, with major laboratories in Columbus, Ohio; Richland, Washington; Geneva, Switzerland; and Frankfurt, Germany.

BNW, a major component of Battelle Memorial Institute, is a well-established, experienced, multidisciplinary contract research organization located in Richland, Washington, with a scientific staff and supporting personnel of more than 4000 employees. Figure VII-I shows the organizational structure of BNW. The Toxicology Department within the Life Sciences Center (Figure VII-2) will have the responsibility for the Philip Morris projects.

Over the past decade, BNW has made a commitment to focusing on inhalation toxicology as a center of excellence within Battelle. As a research facility, BNW believes that its expertise can most effectively be directed to performing studies requiring the inhalation route of administration, and therefore we have directed our attention to refining our capabilities in this area. A large investment has been made by BNW over the past several years in facility modifications, purchase and design of state-of-the-art automated exposure systems, analytical instrumentation, and in training and educating personnel involved in inhalation toxicology studies.

An Inhalation Toxicology Program Office was established within the Toxicology Department to increase the emphasis of the Department on the inhalation toxicology technical and project management areas for increased responsiveness to government and industrial sponsors.

The above factors combine to demonstrate that BNW management, technical and support staff are dedicated to providing the highest quality services and results to the client in the conduct of inhalation toxicology studies at BNW.

B. TOXICOLOGY DEPARTMENT

The Toxicology Department (whose organizational structure is shown in Figure VII-3) is part of the Life Sciences Center. The department has a staff of approximately 120 people working in laboratories and offices covering more then 100,000 ft^2 located in the LSL-II building. Over the past several years, this department has developed a national and international reputation as a center of excellence in inhalation technology and toxicology.

C. PERTINENT EXPERIENCE

Research and development in vapor and aerosol exposure system technology within the Toxicology Department is carried out by a team of scientists, engineers, specialists and technicians. The staff, with overlapping backgrounds in physics, chemistry, inhalation toxicology, toxicologic pathology, radiation biology, computer science and a variety of engineering disciplines, share a basic interest in inhalation technology and have developed and tested exposure systems for animal exposure. Among these developments are two innovative whole-body inhalation exposure chambers (U.S. Patent #4,216,741) and a nose-only inhalation exposure system (U.S. Patent #4,721,060). Other inhalation exposure techniques developed in our laboratory include: steady nebulization of small samples of material; monodisperse aerosol generation from liquids, solids, and vapors; generation of a solid carrier aerosol for oils and chemicals; generation of highly respirable concentrations of fly ash; aerosol generation methods, such as electrostatic generation and nonaqueous nebulization, and an inexpensive method for on-line size fractionation. We have also expanded our capability to measure specific surface, density and dissolution characteristics of aerosol particles.

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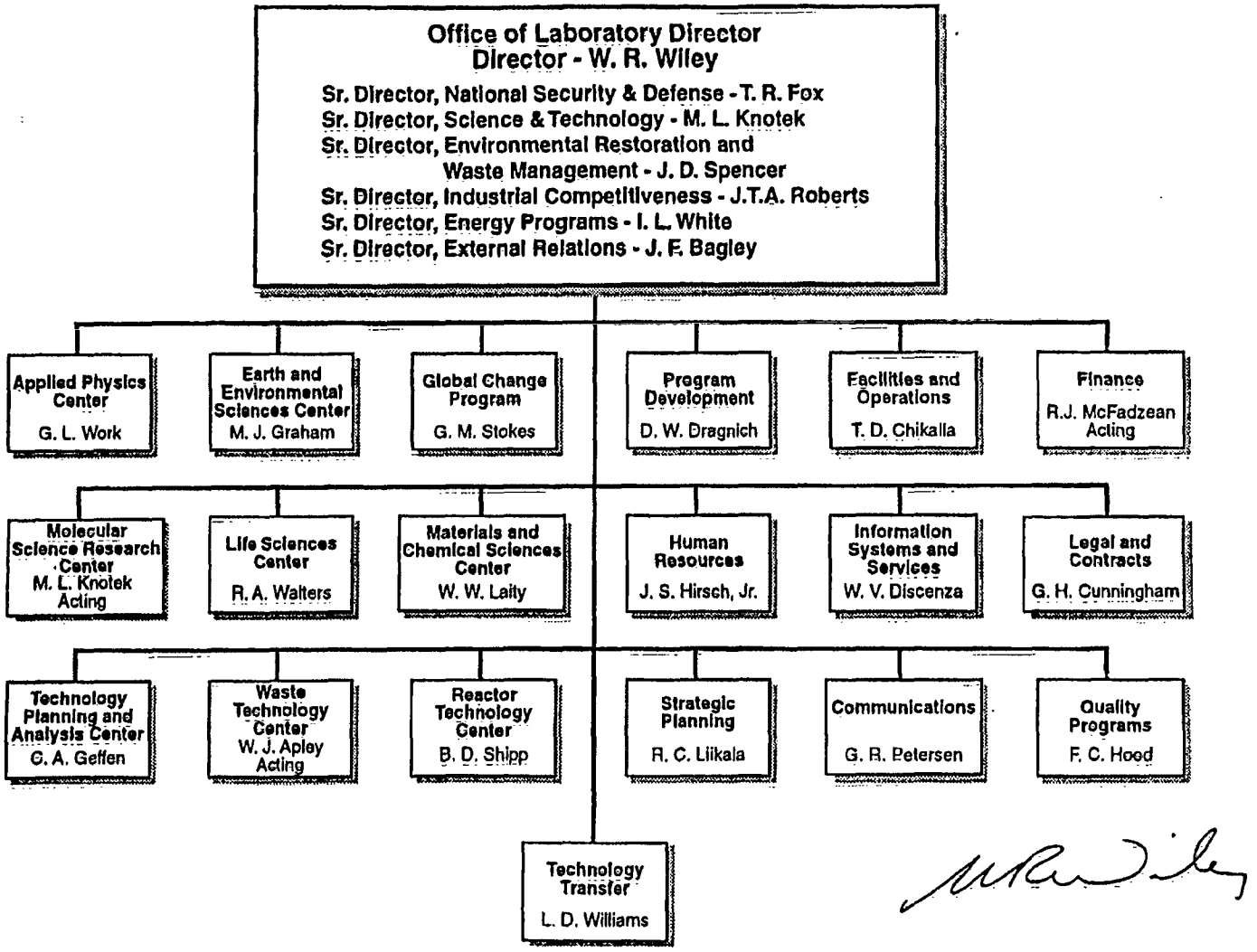

FIGURE VII-1. Organizational Structure of Battelle, Pacific Northwest Laboratories

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BATTELLE-NORTHWEST

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FIGURE VII-2. Organizational Structure of the Life Sciences Center

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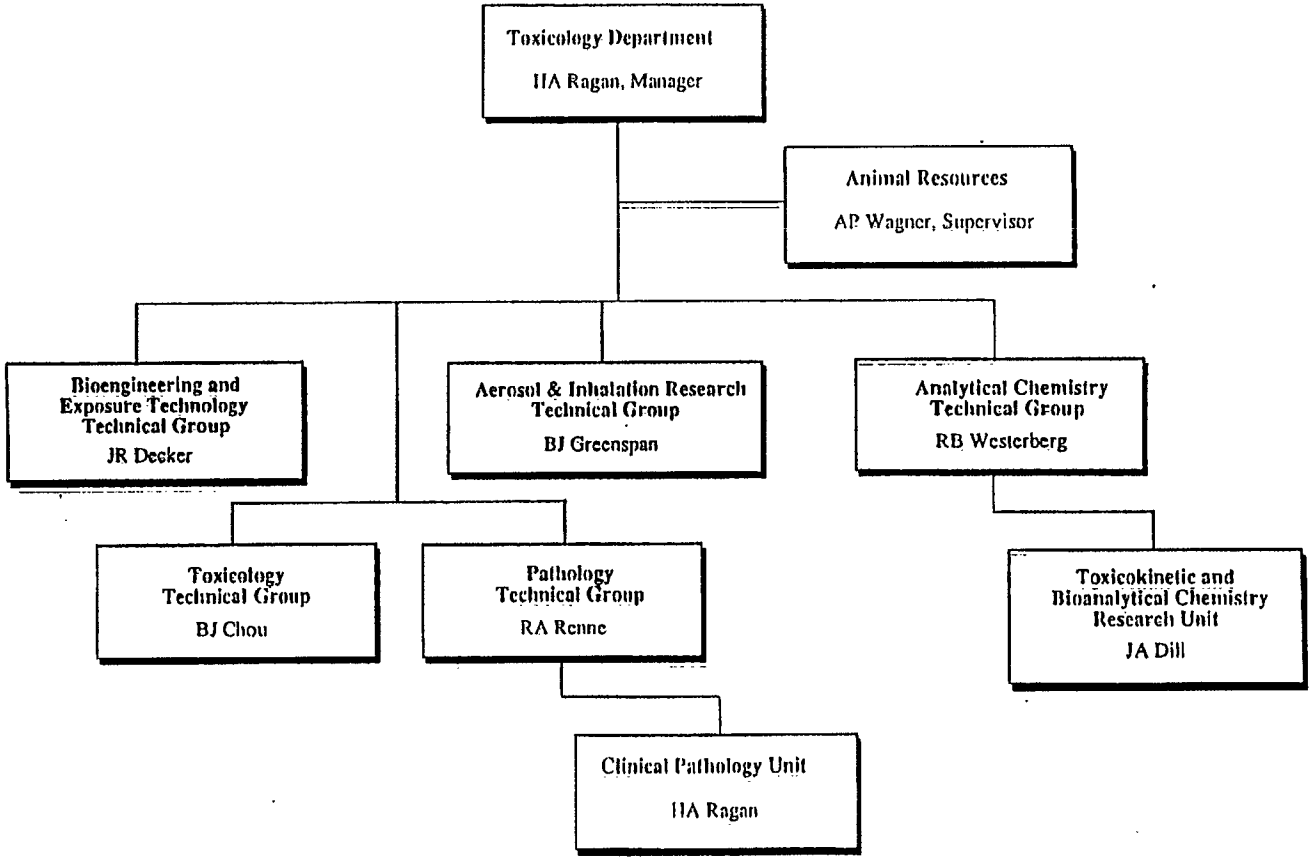

FIGURE VII-3. Organizational Structure of the Toxicology Department

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Scientists at BNW have been conducting animal inhalation studies for more than 25 years. NTP studies of more than 40 chemicals have been or are being investigated for acute, repeated-dose, subchronic, and chronic toxicity, and carcinogenicity in Fischer-344/N rats and B6C3F1 mice. NTP-sponsored inhalation teratology studies in CD-1 mice and Sprague-Dawley rats have been completed on eight chemicals.

Previous inhalation toxicology projects include: inhalation bioassays of cigarette smoke; and health effects of inhaled volcanic ash (National Institute of Environmental Health Sciences; NIEHS); studies in which rats and rabbits were exposed to organic vapors for teratologic studies and others in which hamsters were exposed to fibrogenic dust by intratracheal instillation to determine the role of pulmonary fibrosis in the etiology of lung cancer; development of oil shale aerosol technology (DOE); effects of prolonged inhalation of nickel-enriched fly ash in Syrian Golden hamsters (EPA); pulmonary deposition, translocation, and clearance studies with neutron-activated inhaled dusts; biological effects of inhaled sulfur pollutants (EPA); inhalation hazards to coal miners (DOE); the cross-placental transfer of metallic air contaminants (EPA). Other animal studies have involved pulmonary effects of pellets containing cigarette-smoke condensate implanted in the lungs of rats (National Cancer Institute; NCI).

Inhalation toxicology studies associated with our work in nuclear energy, which are sponsored by the U.S. Department of Energy (DOE), include: 1) aerosol and analytical technology development; 2) cocarcinogenic effects of uranium mine air pollutants (radon daughters, ore dust, cigarette smoke) in rats and dogs to determine the etiology of lung cancer in uranium miners; 3) major programs on the pulmonary and skeletal carcinogenesis of inhaled soluble or insoluble plutonium and other transuranic elements; 4) toxicity of plutonium and other radionuclides in the fetus and neonate; 5) radionuclide metabolism in preexisting disease states; 6) toxicity of noble gases associated with reactor operation, and of elements of the thorium fuel cycle; and 7) the effects of plutonium-sodium aerosols.

BNW has extensive experience with nose-only exposures of rodents as well as other animals including dogs and miniature swine. Rodent nose-only exposures completed in the last 5 years include several cigarette smoke studies, plutonium-238 oxide aerosol, promethium and cesium silicate aerosol, nitropyrene, and glycerol aerosol. The nitropyrene and glycerol exposures were conducted for 6 hours per exposure period. All other exposures required confinement of the animals for shorter periods of time. BNW has developed nose-only exposure technologies that assure fresh exposure material to each animal and assure that there is minimal heat stress to the animals confined in the animal holding tubes.

In addition to our government-sponsored studies, several major inhalation toxicology programs are supported, or have been supported, by private industry. For confidential reasons, the name of the clients and type of studies are not listed.

D. PROJECT MANAGEMENT PLAN

The proposed project will be conducted in BNW's Toxicology Department. The planned project organization (functional) is shown in Figure VII-4. BNW operates with a matrix management system because of the nature of contract research with multiple sponsors and the multidisciplinary approach to research. This system frees the Principal Investiagtors to devote their full attention to project management rather than being diluted by additional concerns required by line management responsibilities.

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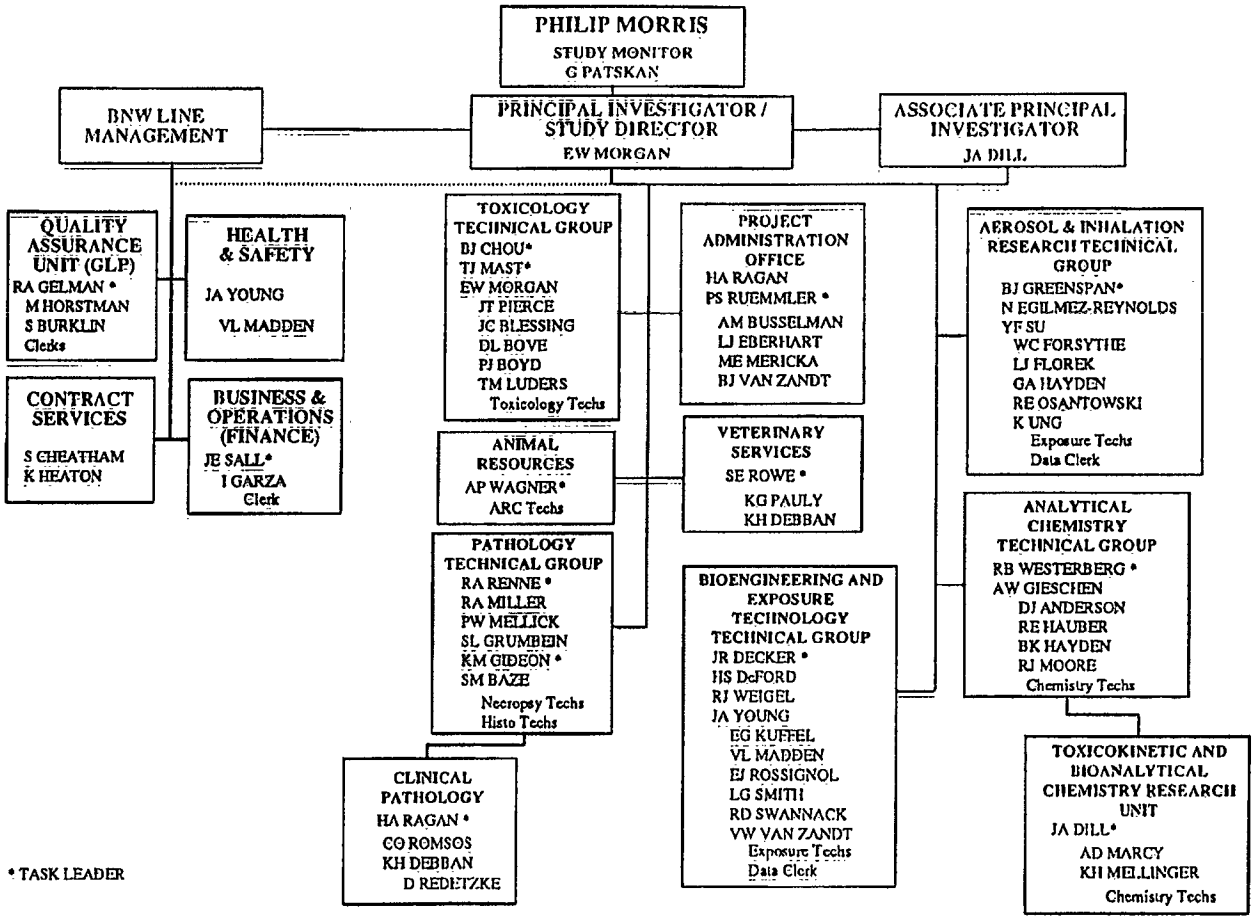

FIGURE VII-4. Organization Structure of the Philip Morris Project

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E. RESPONSIBILITIES AND AUTHORITY OF THE PRINCIPAL INVESTIGATOR

The PI for Philip Morris-sponsored studies, Dr. Morgan, is administratively within the Toxicology Department. Dr. Morgan will be responsible for scheduling, coordinating, monitoring, and reviewing the progress of technical activities, including toxicology, pathology, animal care, veterinary laboratory services, exposure engineering, aerosol physics, analytical chemistry, clinical pathology, and health and safety. He will be the focal point for all program communications within BNW, and will work with both the technical staff and contract group of the Sponsor in coordinating work scopes and changes for the various contracts. Dr. Morgan is also responsible for budgetary and fiscal control of all Philip Morris contracts. He will be responsible for establishing fiscal procedures and monitoring bi-weekly cost accounts and labor summaries provided by the BNW business office.

Within the Philip Morris programs, Dr. Morgan will be in daily communication with the Task Leaders and will hold more formal meetings at least biweekly with the Task Leaders and QA Unit (QAU) to discuss progress, potential technical problems, milestone schedules, and the status of costs versus technical progress.

As PI, Dr. Morgan will have the following authorities and responsibilities of control over all staff members working on Philip Morris projects:

- Direct control, including approval for expenditures of project funds
- Concurrence with the assignment or removal of project staff
- Concurrence with facilities assigned to Philip Morris projects
- Performance evaluations of project staff in their annual staff development reviews by management, including recommendations for promotion
- Recommending, when required, oral and/or written reprimands to staff assigned to the Philip Morris projects
- Reporting unsatisfactory performance of assigned staff to Department Management, and insisting that corrective action be taken
- Approving study protocols, data forms, technical reports, and all other documentation related to the Philip Morris studies
- Specifying appropriate, and timely correction of deficiencies or problems detected by QA and other project staff
- Defining, documenting, and communicating the responsibilities and authorities of staff assigned to the Philip Morris projects
- Assigning supervisory and task leadership responsibilities for weekends, holidays, and vacation periods to assure that qualified professional personnel are available in case an off-normal event should occur
- Conducting an Operational Readiness Review (ORR) with Task Leaders, QA, and Department Management prior to the initiation of animal exposures to include a review of the Study Protocol and SOPs, an analysis of the exposure system, and discussions of potential problem areas or health and safety concerns

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- Conducting frequent meetings with Task Leaders to review compliance with Protocols and SOPs and discuss the various aspects of conducting these studies

- Communicating with the Philip Morris Study Monitor concerning progress, technical concerns, or potential problems.

F. DAILY MONITORING OF THE STUDY EVENTS

As soon as the study protocol is approved and the starting date is determined, the schedule of study events for the Test System (Toxicology, Veterinary Care, Pathology, and Special Toxicology Studies) is compiled into a computer system. Each month the study events will be printed on a monthly calendar form (Figure VII-5), and distributed to the Task Leaders and PI for monitoring and assessing the progress of the study. These schedules will also be used to forecast the staffing requirements. Similar monthly schedules are also prepared for the Test Article (Exposure System, Exposure Generation and Chemical Analysis and Monitoring, Exposure Operations Events, and Chemistry Operations Events).

G. MONITORING PROJECT COSTS AND CONTRACT MODIFICATIONS

As soon as the contract has been awarded, a total project budget and detailed work breakdown structure will be prepared and entered into a computer. Monthly cost reports are used by the PI and Task Leaders to monitor the project costs and commitments. Task Leaders are responsible for providing detailed descriptions for any variances from the original estimates that have not previously been covered by contract changes or modifications.

These variance analysis reports will be used to alert the Sponsor of any additional funding needs as soon as they have been noted.

Contract modifications are automatically assigned an action number by the BNW Contract Associate at the time of receipt. The PI and Department Administrator are alerted of the change and action number on the same day. Cost details and schedule impact are determined by the affected Task Leaders and provided to the Department Administrator who works with the PI, BNW finance and contract personnel in preparing the final proposal. Once a Modification has been defined, the budget, estimated labor hours and schedule details are adjusted accordingly so that all project management information is kept current.

H. MILESTONE SCHEDULES AND DELIVERABLES

A schedule of events and deliverables is prepared at the beginning of each study and entered into a master schedule program maintained by the Department Administrator. Reminders of upcoming events and milestones are sent to all Task Leaders well in advance of the event. Changes in schedules are easily analyzed by this computerized approach, which also provides an effective method of scheduling personnel and facility needs of several projects going on at the same time.

A Phase 1 report will be prepared detailing the construction of the system and prestart testing to define system operating parameters. Interim study reports will be prepared after 12 and 18 months for the chronic study. A final report will be submitted within 12 months of completion of the study. Study report(s) will be prepared for the subchronic and any mechanistic studies performed.

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SAT.	SUN.	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY
1	2	3 Week of Animals to Arrive Body Weights- 1 Day after Arrival	4	5	6	7
8	9	10 Acclimization to Exposure Tubes Health Screen to AR Lab	11 Acclimization to Exposure Tubes Day -6 Body Weights	12 Acclimization to Exposure Tubes	13 Acclimization to Exposure Tubes Randomization Wts, Group Assignments, & Identification	14 Acclimization to Exposure Tubes
15 Acclimization to Exposure Tubes (if needed)	16 Acclimization to Exposure Tubes (if needed)	17 Day 0 Body Weights	18 RASS Male and Female Day 1 Body Weights & Observations RASS First Exposure Day	19 Sham and Diesel Control Day 1 Body Weights & Observations Sham and Diesel Control First Exposure Day	20	21
22	23	24 Week of VAS Animals to AR Lab	25 RASS Week 2 Body Weights & Observations	26 Sham and Diesel Control Week 2 Body Weights & Observations	27	28
29	30	31	April 1 RASS Week 3 Body Weights & Observations	April 2 Sham and Diesel Control Week 3 Body Weights & Observations	April 3	April 4

FIGURE XII-5. Monthly Study Calendar

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The Toxicology Department Archive is a limited access facility within LSL-II. A Senior Administrative Assistant oversees the day-to-day activities of this office. All original source technical records generated on the Philip Morris studies will be filed in this office. The SOP "*Data Handling and Storage of Study Documents and Materials*", covers records management during conduct of the study. At the completion of the Study Report, personnel from the Program Office perform final verification of the completeness and accuracy of the file records. If records are determined to be missing and a thorough search does not locate the data, a missing record form is completed and inserted in the appropriate file folder. A list of the missing records is retained in the Program Office and every attempt is made to locate the missing documents after the rest of the data is forwarded to the Sponsor's archives.

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VIII. QUALIFICATIONS OF PERSONNEL

A. KEY PERSONNEL

Below are brief statements regarding experience and accomplishments of key, critical, and supporting individuals who are germane to the project and their proposed roles for these studies. Complete resumes for key personnel are included in Appendix B. BNW understands the named personnel are necessary for the successful performance of this contract. If substitutions become necessary, BNW will provide a detailed explanation of the circumstances necessitating the substitution to the Sponsor's Study Monitor. The proposed substitutes will have substantially equal abilities and qualifications to those of the person being replaced.

1. Principal Investigator–E.W. Morgan

Dr. Earl W. Morgan received a BS in Agriculture with a science emphasis, and a DVM degree from the University of Missouri in 1973 and 1976, respectively. From 1976 to 1981, Dr. Morgan was in the U.S. Air Force Veterinary Service. From 1981 to 1983, he was in private practice in Missouri and Oklahoma.

In 1983, Dr. Morgan entered the U.S. Army and was assigned to Letterman Army Institute of Research (LAIR) at the Presidio of San Francisco, CA. From 1983 to 1987, he was assigned to the Mammalian Testing Section of the Division of Toxicology, initially as PI and for the last 2 years as the Chief of the section. He was an investigator for toxicological studies conducted in compliance with FDA and EPA Good Laboratory Practices regulations.

From 1987 to 1990, Dr. Morgan was working on a PhD in Toxicology at the University of Kansas Medical Center in the Department of Pharmacology and Toxicology. Dr. Morgan's research project involved the purification and characterization of the function and regulation of two isozymes of a rat liver microsomal carboxylesterase. The major techniques used were microsomal preparation, column chromatography, gel electrophoresis, Western immunoblotting, and enzyme kinetics.

From 1990 to 1992, he served as the Chief of Aerosol Biology, Department of Pathophysiology, U.S. Army Medical Research Institute of Infectious Diseases. Dr. Morgan was responsible for the re-establishment of the aerosol toxicology laboratory at the institute. He provided aerosol support for studies involving toxins, antitoxin therapy, detection of aerosol particles, and efficacy studies for candidate vaccines against airborne exposure to both infectious agents and toxins including high-molecular-weight biological toxins.

Dr. Morgan joined BNW in February 1992. Since that time he has served as Toxicologist and/or Study Director for six studies which use both rodents and canines.

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2. Associate Principal Investigator–J.A. Dill

Dr. Jeffrey A. Dill is a Staff Scientist in the Analytical Chemistry Technical Group of the Toxicology Department. He received a BS in Chemistry from the University of New Mexico (1979) and a PhD in Analytical Chemistry from Colorado State University (1983).

Dr. Dill joined the Toxicology Department in 1987. His duties involve development and evaluation of analytical methodologies used in chemical characterizations and qualification of test atmospheres used

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in inhalation toxicology studies. He is also responsible for the design and conduct of toxicokinetic studies, and for designing and validating assay methods for test chemicals and metabolites in biological samples.

Since joining the Toxicology Department, Dr. Dill has provided technical oversight of the chemistry task on over 20 different inhalation toxicity studies, ranging from 2-week, repeated-dose studies through 2-year chronic studies. These studies have investigated the toxic effects of eight different organic and inorganic test chemicals in rats, mice, or dogs, employing either whole-body or nose-only inhalation exposure units; the majority of which have been aerosol studies. Dr. Dill has completed numerous toxicokinetic or tissue distribution studies, performed in support of inhalation toxicity studies for NTP other governmental and industrial clients.

Prior to joining the Toxicology Department, Dr. Dill was employed by Pacific Northwest Laboratory (PNL) in waste packaging studies for nuclear waste repository investigations. Before joining PNL, he worked for Rockwell Hanford Operations as the Solution Chemistry Laboratory Team Leader. His experience was principally in inorganic analysis of natural water samples, supporting nuclear waste repository investigations.

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B. CRITICAL STAFF

1. Toxicologists–B.J. Chou and T.J. Mast

Dr. Chou, the present Technical Group Leader for Toxicology, will be responsible for assigning and coordinating toxicologists and specialists for the proposed studies. Drs. Mast and Morgan will be responsible for coordinating toxicology procedures, evaluations and reporting.

a. Dr. Billy J. Chou. Dr. Chou received B Vet Sci and DVM degrees from the National Taiwan University, an MS degree in Animal Nutrition and a PhD degree in Physiology from Kansas State University (KSU). He is a Diplomate of the American Board of Toxicology with more than 20 years of experience in industrial toxicology. He has extensive experience in safety assessment of industrial chemicals. These include the design, supervision, evaluation and reporting of the toxicity and target organ toxicity involving industrial chemicals and therapeutic agents in laboratory animals. Before joining Battelle, Dr. Chou was the Department Head of Toxicology and Animal Facilities at William H. Rorer, Inc. He was responsible for conducting and reporting all required animal toxicity studies for Investigational New Drug (IND) and New Drug Approval (NDA) submissions. He was also the Project Manager for "growth promotion" and "anticoccidial" projects. Dr. Chou joined BNW in November 1984 as the National Toxicology Program (NTP) toxicologist and assistant PI and assumed the PI position in January 1986. Since then, Dr. Chou has been the PI for 26 NTP chemicals.

b. Dr. Terryl J. Mast. Dr. Mast has a BS degree in Biochemistry and Biophysics, an MS in Agricultural Chemistry, and a PhD in Environmental Chemistry through the Environmental Toxicology Department, University of California (UC), Davis. In addition, she was a postdoctoral researcher in Developmental and Reproductive Biology from 1984 through 1985 at the California Primate Research Center at UC, Davis. She is currently a Staff Scientist in the Toxicology Department at BNW.

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Dr. Mast joined BNW in 1986. Her research interests, in addition to general inhalation toxicology, are in the area of developmental and reproductive toxicology, mechanisms of developmental toxicity, and distribution, and pharmacokinetics of toxicants.

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Dr. Mast functions as Study Director and toxicologist for some NTP projects at BNW and also serves as Task Leader for the sperm morphology and vaginal cytology evaluation (SMVCE) task. She is PI for the current effort on nose-only inhalation studies being conducted for a confidential client.

Dr. Mast joined BNW following 2 years of work in Developmental Toxicology at the California Primate Research Center, where she studied the teratology, pharmacokinetics, and placental transfer of valproic acid in nonhuman primates and rodents administered by oral gavage. Her interest in metabolism and maternal-fetal pharmacokinetics led to studies on the development of hepatic xenobiotic-metabolizing enzymes in the fetal monkey. She conducted an oral gavage study in rats to correlate teratological effects of the pesticide oftanol, with maternal plasma kinetics, cholinesterase activity, and metabolism during organogenesis. She designed and participated in a study correlating plasma levels and toxic effects of dibromodichloropropane (DBCP) administered to Rhesus monkeys in drinking water on the male reproductive system.

2. Pathologists--R.A. Miller, S.L. Grumbein, R.A. Renne, and P.W. Mellick

Dr. Miller will be responsible for the necropsy supervision and histopathologic evaluations of all mice on the proposed studies.

a. Dr. Rodney A. Miller. Dr. Miller received his DVM degree, with distinction, from Iowa State University in 1971. After 4 years of clinical practice, he was selected for a PhD program at Washington State University, where he obtained 4.5 years of experience in diagnostic pathology in the Washington Animal Disease Diagnostic Laboratory. Much of his diagnostic experience involved tumor diagnosis in a variety of domestic animals and rodents. His thesis research was concerned with the inter-relationship of diabetes and atherosclerosis in rabbits. After receiving his PhD from Washington State University in 1979, he joined BNW, where he has participated in a number of rodent studies, including long-term effects of inhaled acid aerosols in rats, late effects (carcinogenesis) of americium nitrate in rats, and rat mammary carcinogenicity of injected plutonium citrate. Dr. Miller has participated in several subchronic and chronic inhalation studies in rats and mice exposed to various solvent-refined coal products. Dr. Miller successfully passed the certifying examination of ACVP in September 1980. Dr. Miller has 12 years of experience in necropsy supervision and in histopathologic evaluations of tissues from rodents in prechronic and chronic inhalation studies for NTP. The many NTP-sponsored inhalation studies which Dr. Miller has evaluated include the prechronic and chronic inhalation studies of 1,3-butadiene and chloroprene, and the prechronic studies on isoprene, gallium arsenide, and gallium oxide. Dr. Miller will be responsible for necropsy supervision and histopathologic interpretation of tissues for these studies on this project.

b. Dr. Sondra L. Grumbein. Dr. Grumbein received her DVM degree from KSU in 1971. Following 2 years of clinical practice, she returned to KSU and completed a PhD program in Veterinary Pathology in 1977. During that time Dr. Grumbein was Veterinarian for the KSU Animal Resources Facility and monitored research animals and facilities campus-wide. After receiving her PhD, Dr. Grumbein joined the Pathology Faculty of the College of Veterinary Medicine with responsibilities in teaching, diagnostic service, and research.

In January 1979, Dr. Grumbein joined the faculty at the newly formed School of Veterinary Medicine at Oregon State University (OSU). She developed the general pathology course taken by first-year veterinary students in their third quarter, including both microscopic and gross pathology laboratories, and taught that course to the first three classes admitted to the OSU program. Dr. Grumbein also developed and taught an animal pathology course for non-veterinary students. The remainder of her time was spent in the Oregon Veterinary Diagnostic Laboratory, providing diagnostic services to clinical veterinarians as well as livestock, poultry, and pet owners. She was certified as a Veterinary Pathologist by the American College of Veterinary Pathologists (ACVP) in 1979.

REDACTED Dr. Grumbein joined Battelle Columbus Laboratories (BCL), Columbus, OH, as Associate Manager of the Pathology Section. One year later she became Chief of Pathology, and in 1985,

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Manager of the Pathology Section, responsible for anatomic and clinical pathology operations, including 4 veterinary pathologists and 25 histology and clinical pathology technical staff. The majority of that operation's work consisted of rodent studies performed for NTP under GLP and other government sponsors. Dr. Grumbein was involved in proposal preparation and costing, as well as protocol development and scheduling. She reviewed the work of BCL pathologists and established the group's reputation for producing pathology data generally uncluttered by duplicate diagnoses and spurious trends. Dr. Grumbein also supervised necropsies and read numerous NTP prechronic and chronic rodent studies, utilizing either the TDMS computer system or XYBION for data capture and management.

In 1987, Dr. Grumbein joined Pathology Associates, Inc., (PAI) as Director of the North Carolina Division, and took over as PI of their pathology support contract with NTP. In that role she interacted directly with NTP Project Officers and pathologists to meet their needs under that contract. Dr. Grumbein managed a full-service histology laboratory in support of NTP, as well as an electron microscopy laboratory with both transmission and scanning capabilities. She chaired numerous NTP Pathology Working Groups for both prechronic and chronic studies. Dr. Grumbein completed several special pathology projects for NTP, the largest of which included review of slides from ~2,000 rats and 1,100 mice retrieved from NTP Archives. The results of reevaluation of the incidences of selected neoplasms in those rodents, along with other measurable factors such as size and number of section, were included in two papers published in *Toxicologic Pathology* by NTP. Other special pathology projects included reading step-sections of residual kidneys of male rats on a chronic study for proliferative lesions and reading sections of residual pancreas of rats for exocrine lesions. Dr. Grumbein also performed or supervised a number of reviews of residual wet tissues for untrimmed potential lesions.

In January 1990, Dr. Grumbein rejoined Battelle at its Pacific Northwest Laboratories and has since participated in two repeated dose, three subchronic, and four chronic NTP inhalation studies, including supervision of necropsies and histopathologic evaluation of tissues from rats on the subchronic inhalation study of glutaraldehyde and the chronic inhalation study of chloroprene. Dr. Grumbein has 10 years of experience in necropsy supervision and histopathologic evaluations of tissues from prechronic and chronic NTP rodent studies.

c. Dr. Roger A. Renne: Dr. Renne, the Technical Group Leader for Pathology in the Toxicology Department, is responsible for the coordination of the pathology tasks, including animal sacrifice, necropsy, histology, and histopathology. He holds a DVM from Washington State University, and is a Diplomate of ACVP. He has over 21 years of experience in the pathologic evaluation of tissues from experimental animals used on chronic toxicity and carcinogenesis studies.

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Dr. Renne has been PI on several projects, including studies evaluating the fibrogenic and carcinogenic potential of particulate oil shale materials using repeated intratracheal instillation, and inhalation toxicity studies of H_2S and ammonia in rodents. He has also been responsible for histopathologic evaluation of pulmonary lesions in dogs exposed to cigarette smoke, and gross and microscopic evaluation of tissues from dogs exposed to carcinogenic doses of particulate radionuclides via inhalation, and gross and microscopic pathology of hamsters exposed to fibrogenic particulates. Dr. Renne was PI for an industry-sponsored dermal carcinogenesis project and was acting PI for the NTP project for 14 months. Dr. Renne has 14 years of experience in necropsy supervision and 18 years experience in histopathologic evaluation of tissues from rodents in the NCI/NTP carcinogenesis program.

d. Dr. Paul W. Mellick: Dr. Mellick received his DVM degree from The Ohio State University in 1962 and his MS degree in Veterinary Pathology from New York State Veterinary College at Cornell University in 1964. After 2 years of clinical practice, he entered the Veterinary Corps of the U.S. Army. After a 1-year tour of duty in Vietnam during which he established a Veterinary Diagnostic Laboratory for Vietnamese livestock and poultry, he returned to the Armed Forces Institute of Pathology where he completed residency training in veterinary pathology. He was certified as a Veterinary Pathologist by ACVP in November 1970. From 1970 to 1972, he served as Chief, Toxicology Division, U.S. Army Environmental

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Hygiene Agency, Edgewood Arsenal, MD where he conducted toxicology studies on chemicals important to the Army using all routes of administration including inhalation, and a variety of animal models including rodents, dogs, birds and primates.

In 1972, he entered the PhD program in Comparative Pathology at UC, Davis. His thesis research involved inhalation toxicology and pulmonary pathology in nonhuman primates exposed to ambient concentrations of oxidant air pollutants. After the completion of his graduate studies in 1975, Dr. Mellick returned to his military duties as Chief of Pathology at the Letterman Army Institute of Research, Presidio of San Francisco. During his 8 years in this assignment he directed and conducted pathology research on a variety of biomedical research projects in the fields of toxicology, safety and efficacy of blood replacement solutions, nutrition and experimental surgery. He organized and developed Letterman's toxicology research and testing program, the only GLP-compliant toxicology capability in the U.S. Army. He organized and directed a highly successful residency program in veterinary pathology. In 1983 and 1984 he served as Head, Veterinary Medicine Department, U.S. Naval Medical Research Unit #3, Cairo, Egypt, where he planned, directed and conducted research on infectious zoonotic diseases in Egypt and other Middle Eastern countries. He then returned to the Presidio of San Francisco and became the Deputy Commander, Letterman Army Institute of Research. In this assignment he planned, directed and managed scientifically diverse biomedical research programs with a staff of 250 and an annual budget of $14 million. Concurrently, he served as Veterinary Pathology Consultant to the Surgeon General, assessing veterinary pathology requirements for all Department of Defense laboratories worldwide; monitored training, career development and professional capability of approximately 60 military veterinary pathologists and made recommendations concerning appropriate veterinary pathology staffing to the Chief of the U.S. Army Veterinary Corps.

In 1987, Dr. Mellick retired from active military service to pursue interests in inhalation toxicology and pulmonary pathology at BNW. Since joining BNW, his primary responsibilities have been necropsy supervision and histopathologic evaluations of tissues from rodents in prechronic and chronic inhalation studies for NTP, and PI for inhalation toxicology studies for an industrial sponsor. He was the study pathologist for NTP-sponsored subchronic inhalation toxicity studies of gallium arsenide and isoprene in rats and glutaraldehyde in mice.

3. Clinical Pathologist--H.A. Ragan

Dr. Harvey A. Ragan is the Manager of the Toxicology Department, as well as the Director of the Clinical Pathology Laboratory within the department. Dr. Ragan has degrees in biology and in veterinary medicine, and has postdoctoral training in hematology. His special research interests are the interrelationships between pollutant metals and nutritional deficiencies on the hematopoietic and immune systems, the clinical pathologic effects of electromagnetic fields or internal emitters on experimental animals, and the *in vitro* quantitation of effects of pollutants on hematopoietic stem cells. Dr. Ragan has over 25 years of experience in laboratory medicine applied to beagle dogs, miniature swine, miniature goats, and laboratory rodents.

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Dr. Ragan served as the PI/toxicologist for the NTP projects from February 1984 until December 1985, and has over 12 years of experience with NTP-sponsored toxicity studies. Dr. Ragan will serve as the clinical pathologist on the proposed projects.

4. Chemists--R.B. Westerberg, A.W. Gieschen, C.W. Wright, R.N. Lee, and S.D. Harvey

a. Dr. R. Bruce Westerberg. Dr. Westerberg is the Technical Group Leader for Analytical Chemistry. He received his PhD in Analytical Chemistry from Purdue University in 1974. He has a broad background and experience in bioanalytical chemistry, and characterization of test atmospheres in exposure chambers. As the Technical Group Leader for the Analytical Chemistry Group of the Toxicology

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Department, he is responsible for supervising personnel performing analytical and bioanalytical measurements.

Dr. Westerberg has acted as PI for a series of programs in which nicotine and cotinine were determined in plasma samples. He has also participated in Department of Energy (DOE) studies concerning the metabolism of complex mixtures. He has been chemistry task leader for numerous inhalation studies of a wide variety of test material including tobacco smoke, humectants, pharmaceuticals, various inorganic and organic aerosols, and numerous organic gases and vapors.

Before joining BNW, Dr. Westerberg was employed by E.I. DuPont Chambers Works, Jackson Laboratory, as an Analytical Research Chemist (1974-1980). During the early part of that period, he was responsible for the laboratory's HPLC facilities. Later he assumed responsibility for upgrading and operating the laboratory's GC/MS facilities. This work included quantitative analysis of industrial waste waters for trace levels of EPA-priority pollutants, and analysis for polychlorinated biphenyls (PCB) in complex organic materials. Dr.Westerberg is a member of the American Chemical Society (Division of Analytical Chemistry), the American Association of Pharmaceutical Scientists and the American Society for Mass Spectrometry.

b. Andrew W. Gieschen. Mr. Gieschen is a Senior Research Scientist involved in analytical organic chemistry in the Analytical Chemistry Group. He has a BS degree in Microbiology and Chemistry from Arizona State University (1983) and a MS degree in chemistry from Washington State University (1986).

He is an experienced chemist with expertise in trace organic analysis using high performance liquid chromatography and capillary gas chromatography. He has experience with GC/FID, GC/ECD, GC/TCD, GC/MS, HPLC/UV, and HPLC/Fluorescence techniques for analyses of environmental samples and samples obtained from biological matrices. Mr. Gieschen has in-depth knowledge of personal computer operating systems and electrical engineering. He also has a strong background in organic chemistry, organic synthesis, Fourier transform infrared spectroscopy, nuclear magnetic resonance spectroscopy, thin-layer chromatography, and low-pressure liquid chromatography.

Since joining the Toxicology Department in 1987, Mr. Gieschen has had the responsibility of supporting several NTP projects. His responsibilities include development, evaluation, and implementation of analytical methods for chemical characterization and quantification of inhalation exposure test atmospheres. He is also involved in the designing, validation, and implementation of analytical techniques used for bioanalytical and toxicokinetic studies. Since joining the BNW, Mr. Gieschen has been involved in the leadership and implementation of the analytical chemistry methods for over 20 inhalation toxicity studies involving more than 10 different chemicals. These studies have measured the toxicity of these organic compounds in rats and mice using whole-body and nose-only exposure units. Mr. Gieschen has also been responsible with the development of a highly automated HPLC analytical laboratory and implementation of a computerized automated on-line monitoring system.

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c. Cherylyn W. Wright. Ms. Wright is an Analytical/Organic chemist specialized in sampling, extraction, and analysis techniques for trace organic analyses of solid, liquid, and gaseous sample matrices. She is an experienced separations chemist with expertise in gas and liquid chromatography with special emphasis in high-resolution gas chromatography using fused silica capillary columns. She has experience in the use of element-specific detection, as well as gas chromatography/mass spectrometry (GC/MS) and GC/Fourier transform infrared detection (GC/FTIR) for characterizing complex mixtures and compounds of interest from energy-related and environmental samples. In her graduate work, she developed a scheme to separate and identify sulfur-heterocycles from complex coal-liquid and shale-oil matrices.

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Since coming to Battelle, Ms. Wright has had experience in the separation, identification, and quantification of heteroatomic organic species in complex synthetic fuels, petroleum products, shale oils, diesel particulates, organic hazardous waste samples, aqueous leachates of organic matrices, shale waste waters, air pollution samples, and contaminated site environmental samples. She has directed the separation of coal-derived materials by chemical class fractionation for biological testing, as well as characterizing the class fractions chemically. In addition, she has directed the development of extraction and analysis procedures for specific compounds of interest from biological matrices such as urine, saliva, and plasma. This effort included analysis of plasma samples for nicotine and cotinine via fused silica capillary column gas chromatography with a nitrogen-specific detector. She has developed methods to monitor the stability and degradation of energy-related complex mixtures as well as specific compounds of interest used in inhalation toxicology studies. She has also been involved in the development of innovative sampling, extraction, and separation techniques for organic analysis including large volume air and water sampling, laboratory and in-the-field supercritical fluid extraction, and capillary zone electrophoresis.

d. Dr. Richard N. Lee. Dr. Lee has participated in the design and implementation of sample collection and analysis tasks conducted in support of several inhalation studies. This work has included a key role in the development of analytical procedures for the determination of trace levels of NO_X in an inhalation study involving co-administration of ozone and the tobacco-specific nitrosamine, NNK.

Dr. Lee has been trained as an inorganic chemist and has over 21 years experience in conducting trace gas and aerosol measurements in the ambient atmosphere. His experience includes the utilization of both continuous and integrated sampling techniques for the measurements of airborne materials. He has also conducted precipitation chemistry studies which have involved the measurement of inorganic and organic species in precipitation. These studies have focused on the acidic precipitation problem and the removal of hazardous organic pollutants, such as nitrobenzene and methylchloroform by rain. He has also developed and used special monitoring capabilities for measuring atmospheric tracer gases, hydrogen peroxide and volatile sulfur compounds in the atmosphere. Recently, he has been involved in trace organic analysis by GC/MS and sample preparation using supercritical fluid extraction.

e. Dr. Scott D. Harvey. Dr. Harvey joined Battelle after completion of his PhD degree the summer of 1988. Having pursued advanced training under two Tswett Medalist chromatographers, Professor R.E. Sievers (University of Colorado) and Professor M.V. Novotny (Indiana University), his primary interests reside in the application of high-resolution separation techniques for the isolation and identification of compounds in complex matrices.

Dr. Harvey's current interests center on the further development and application of high-resolution microcolumn HPLC, micellar electrokinetic capillary chromatography, and electroosmotically driven microcolumn HPLC techniques for studying the impact of environmental pollutants on biological systems. Specific interests include the delineation of metabolism associated with the detoxification of xenobiotic pollutants in plant systems and the ultratrace analysis of DNA adducts that result from environmental exposure to carcinogens. Carbon-fiber amperometric and semiconductor laser-induced fluorescence detectors are under development to allow attomole detection of DNA adducts. Related pursuits include microcolumn HPLC isolation of a variety of natural products arising from plant secondary metabolism (alkaloids and taxanes). These endeavors include multidimensional techniques and strategies for the resolution of enantiomeric forms which are often encountered in metabolic products. Dr. Harvey will participate in the protein/DNA adduct studies.

5. Exposure Technology Personnel—J.R. Decker, B.J. Greenspan, H.S. DeFord, and N.E. Egilmez-Reynolds

a. Mr. John R. Decker. Mr. Decker is the Technical Group Leader of the Bioengineering and Exposure Technology Group in the Toxicology Department. He holds a BS degree in Electrical Engineering from Colorado State University. Since joining Battelle in 1969, he has been

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instrumental in the development of a multitude of generation and monitoring systems for inhalation toxicology studies of aerosols, vapors, and gases; including cigarette smoke, radionuclide aerosols, fly ash, volcanic ash, military obscurants, pharmaceutical aerosols, diesel exhaust, and a multitude of organic vapors and inorganic aerosols. He has developed systems for monitoring and controlling the environment of animal exposure chambers. He has also developed several pulmonary function analysis instruments especially for small-animal studies. He contributed to the development of both the whole-body and nose-only exposure chambers presently used at Battelle for inhalation toxicology studies.

Mr. Decker engineered the automated data acquisition and control systems that monitor and control the basic inhalation test system functions including chamber airflow, vacuum, temperature, relative humidity, and chemical concentration.

b. Dr. Bernard J. Greenspan. Dr. Greenspan is the Technical Group Leader for the Aerosol and Inhalation Research Group in the Toxicology Department. He holds a BA with honors in Physics from Clark University and an MS and PhD in Biophysics from the University of Rochester Medical Center. Since joining BNW in 1985, he has been the exposure task leader for numerous aerosol and vapor inhalation exposures for NTP and pharmaceutical clients.

In addition to his work on inhalation toxicology, Dr. Greenspan is also involved in the design of new generation and delivery systems for pharmaceutical aerosols.

Prior to joining the staff at Battelle, Dr. Greenspan studied the toxicity of lithium combustion aerosols and the deposition and clearance of beryllium aerosols at the Lovelace Inhalation Toxicology Research Institute in Albuquerque, New Mexico.

From 1981 to 1983, he was a Staff Scientist in the Inhalation Department of Union Carbide Corporation's Bushy Run Research Center in Export, Pennsylvania. He was responsible for the design of aerosol and vapor exposure systems.

c. Mr. Henry S. DeFord. Mr. Henry S. DeFord is currently the Bioengineering and Exposure Technology Group Inhalation Exposure Engineer in the Toxicology Department. He holds a BS degree in Microbiology from the University of Washington and has studied engineering at Washington State University. Since joining Battelle in 1976, he has worked on the development of many test article generation and monitoring systems for inhalation toxicology studies of aerosols, vapors, and gases. He has designed components for monitoring and controlling the environment of animal exposure chambers. He has also been involved in the development of proprietary equipment and provides engineering support for non-inhalation research projects.

Mr. DeFord's experience includes first-line supervision of daily exposure tasks as well as supervision of the design, purchase, and construction of exposure system components. He has worked closely with Mr. Decker in the design and development of inhalation exposure control systems at BNW.

d. Dr. Nazli Egilmez-Reynolds. Dr. Egilmez-Reynolds joined BNW in 1992 as a Senior Research Scientist in the Aerosol and Inhalation Research Group in the Toxicology Department. She holds a BE in Chemical Engineering from Aegean University in Izmir, Turkey, and a MSc and PhD in Chemistry (specialized in Aerosol Science) from Essex University in Colchester, England. She was also employed as a research worker for two years in Essex in the Aerosol Laboratory of Prof. C.N. Davies where she developed and characterized a nanometer aerosol generator and performed coagulation experiments using monodisperse and mixed aerosol systems. Her work developed the theory of coagulation of fine particles which was published in the Proceedings of Royal Society of London in 1982.

From 1983 to 1987, she worked as Research Scientist in the Product Research Group of the British-American Tobacco Company, Research and Development Center in Southampton, England. She was

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responsible for the Aerosol Laboratory and acted as the Chief Aerosol Scientist for the BAT group worldwide, coordinating research with associated laboratories in Louisville (US), Hamburg (Germany) and (Montreal) Canada. She developed and executed work programs on aerosol generation and behavior inside cigarettes, filters and human upper airways and participated in the Environmental Tobacco Smoke studies.

She worked as an independent Consultant on Aerosol and Tobacco Smoke between 1987 to 1992 and taught Chemistry as a Visiting Assistant Professor in the Chemistry Department of the University of Louisville between 1988-1991.

Since joining BNW last October, she has been the exposure task leader for three aerosol exposure studies.

6. Inhalation Technology

a. Inhalation Exposure Specialists. The Inhalation Exposure Specialists aid in design, supervise construction, and aid in test and evaluation of both hardware and software for inhalation exposure systems. They aid in generation, verification, validation, and the documentation for these systems. They train; supervise and schedule the activities of the Exposure Technicians in the routine operation, maintenance, and calibration of the exposure systems and in quality control activities. They are responsible for the daily review of exposure and environmental data and for assuring that Good Laboratory Practices are followed in the conduct of data collection. The Exposure Specialists aid in the compilation of data reports and in preparing technical input for proposals and scientific publications.

Name	Title	Years of Experience
LJ Florek	Inhalation Exposure Specialist	10
WC Forsythe	Inhalation Exposure Specialist	13
EJ Rossignol	Inhalation Exposure Specialist	19
RE Osantowski	Inhalation Exposure Specialist	1
K Ung	Inhalation Exposure Specialist	1

b. Engineering Specialists

The Engineering Specialists aid in the detailed electrical and mechanical design, supervise construction, and aid in test and evaluation of the exposure data acquisition and controls systems. The engineering specialists write, document and verify software for data acquisition and data analysis; provide routine maintenance and troubleshooting; prepare SOPs, and contribute to reports, proposals, and scientific publications.

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Name	Title	Areas of Responsibility	Years of Experience
EG Kuffel	Electronics Specialist	Design, fabrication, testing, maintenance of automated data acquisition systems for all NTP studies.	20
RD Swannack	Computer Program Specialist	Computer programming for data acquisition and data analysis for NTP studies.	11
VW Van Zandt	Computer Program Specialist	Computer programming for data acquisition and data analysis for NTP studies.	11
GA Hayden	Mechanics Specialist	Design, fabrication, testing, maintenance of exposure mechanical systems.	6

c. Inhalation Exposure Technicians

The Inhalation Exposure Technicians are responsible for the daily control of the test article exposure; routine maintenance and calibration of the exposure equipment; and routine recordkeeping according to Good Laboratory Practices.

Name	Title	Years of Experience
GR Ell	Inhalation Exposure Technician	13
EM Milliman	Inhalation Exposure Technician	23
RD Devoir	Inhalation Exposure Technician	3
RV Lilienthal	Inhalation Exposure Technician	3
R Rodriquez	Inhalation Exposure Technician	3
KF Tarr	Inhalation Exposure Technician	6
RP de la Bretonne	Inhalation Exposure Technician	1
NZ Hertelendy	Inhalation Exposure Technician	1
CK Mullally	Inhalation Exposure Technician	1
DL Bowers	Inhalation Exposure Technician	1
SF Conley	Inhalation Exposure Technician	1

7. Laboratory Animal Medicine--S.E. Rowe

Dr. Stephen E. Rowe has a BS degree in Agriculture (biochemistry option) and a DVM degree from Purdue University, and an MS degree in Pathology from the University of Michigan. He is a Diplomate of the American College of Laboratory Animal Medicine and has 25 years of experience in laboratory animal medicine; 20 of those years have been at BNW. Prior to that, he completed a 2 ½ year training program in laboratory animal medicine at the University of Michigan where he received training and experience in the diagnosis of rodent diseases by clinical observation, by gross and histopathologic examination, and by laboratory culture and testing. Before that, he worked as an investigator on a variety of toxicology studies and directed animal care operations at the U.S. Army Environmental Hygiene Agency.

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Dr. Rowe initiated a capability in clinical microbiology at BNW in 1975 and has supervised this function since that time. He has had the responsibility for and much experience in the preparation of barrier procedures for the exclusion and control of infectious agents. During his 20 years at Battelle, he has provided guidance and oversight for the varied animal care and use activities at the Laboratory.

Dr. Rowe will closely monitor the health of the animals assigned to these studies and will interact with the PI, the Animal Resources and Toxicology Staff, and other personnel assigned to the studies to assure that feed and water quality, sanitation, barriers and environmental conditions are properly maintained. He will also monitor the quality of animal care activities and ensure compliance with animal welfare regulations.

8. Health and Safety Officer--J.Y. Young

Mr. John Y. Young received his BS degree in Preventive Medicine in 1971 and his MS degree in Public Health (Industrial Hygiene) in 1980, both from the University of Washington, Seattle, WA. Mr. Young is a Diplomate of the American Academy of Industrial Hygiene, certified in the Comprehensive Practice of Industrial Hygiene; a Diplomate of the American Academy of Sanitarians, and is a Registered Sanitarian with both the National Environmental Health Association and the Board of Registration of Sanitarians in Pennsylvania.

Before joining Battelle, Mr. Young had spent 20 years with the U.S. Army as an Environmental Science Officer specializing in industrial hygiene.

Mr. Young is responsible for the planning and execution of an Environmental, Safety, and Health (ES&H) Program in support of the research contracts related to chemical exposures. Mr. Young represents the Toxicology Department on ES&H issues, and ensures that all research activities are in compliance with OSHA, EPA, and other relevant regulations. Additionally, Mr. Young is also involved in individual research projects within BNW, working on toxicologic evaluation and exposure assessment of potentially hazardous vapors in underground storage tanks on the Hanford site.

Mr. Young will oversee the proper planning and execution of an ES&H program in support of the proposed research program, and provide input to research scientists in industrial hygiene evaluation and control, workers' protection, and environmental pollution prevention.

9. Quality Assurance Officer--R.A. Gelman and M.G. Horstman

Ms. R. Ann Gelman supervises the activities of the QA Unit. She reports directly to the Manager of Operations of the Life Science Center.

a. R. Ann Gelman. Ms. Gelman received her B.S. degree in Foods and Nutrition from Carnegie Institute of Technology in 1960 and an MS degree in Nutrition in 1965 from the University of Pittsburgh. In her present position (QA Unit Specialist, GLP Office), she is responsible for ensuring that projects conducted under GLP regulations for nonclinical laboratory studies are in compliance with those regulations. She is also responsible to management for: 1) ensuring that the research facility is in compliance with GLP regulations, 2) auditing and surveillance activities, 3) assisting Project Managers in setting up GLP procedures and documentation for their projects, and 4) assisting auditors from clients or other agencies during facility and project inspections.

Prior to assuming the QA Unit Senior Specialist position, Ms. Gelman was, for 5 years, a Research Assistant in the Biology and Chemistry Department, assisting in studies of the toxicology of inhaled fossil-fuel-related carcinogens. Previous to that, she was a Senior Research Assistant for 8 years, supervising a group specializing in the biochemistry of trace lipids. Ms. Gelman has 12 years of experience reviewing toxicity studies conducted under the GLP regulations.

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b. Michael G. Horstman. Mr. Horstman earned B.S. degrees in Zoology and Biology in 1979, and an M.S. in Pharmacology and Toxicology in 1987. Mr. Horstman was a Scientist in the Toxicology Group within the Toxicology Department for 4 years. He had previously worked 2 years as a Technical Specialist in the Biology and Chemistry Department at BNW. He has extensive laboratory experience in mammalian toxicology and the study of molecular mechanisms of toxicity, particularly metabolic activation and macromolecular adduction processes. This also includes experience in a variety of laboratory analytical methods.

In his present position, Mr. Horstman helps to assure management that the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with GLP regulations. His duties include surveillance of study procedures, data audits, reviews of Final Reports, as well as assisting the Lead QA Officer during facility and project inspections from clients and other agencies.

C. OTHER SENIOR PERSONNEL

1. Dr. Richard P. Schneider

Richard P. Schneider obtained his B. S. in Biology from Kent State University and his Ph.D. in Animal Physiology from Washington State University in 1968. He has been the manager of the Molecular and Structural Biology Section since 1980. In this capacity he has 13 years of experience in carcinogenesis and mutation research. For the past 6 years he has been using molecular biology methods to identify mutation in oncogenes and tumor suppressor genes. This includes all of the methods needed for the Blue Mouse assay including: DNA extraction, bacteria and λ phage culture, phage plaque selection, molecular cloning in phagmids, polymerase chain reactions and DNA sequencing. Dr. Schneider is well qualified to lead this task.

2. Dr. David L. Springer

Dr. Springer has a BS in General Studies (Biology and Chemistry) and a PhD in Biochemistry from the University of Idaho. Additional training as a Research Associate (post-doc) was obtained from the Department of Agricultural Chemistry at Oregon State University. Dr. Springer joined BNW in 1979 and is a Staff Scientist in the Biology and Chemistry Department. Dr. Springer is a Diplomate of the American Board of Toxicology and a member of the Society of Toxicology.

Dr. Springer has 15 years experience in biochemical toxicology with emphasis on the mechanisms whereby organic materials contribute to tumor development. This experience includes metabolism, metabolite isolation and structural characterization, the enzymology of Phase I and Phase II reactives, and DNA/protein adduct isolation and characterization. Currently, Dr. Springer is funded by DOE/OHER to study the influence of bulky chemical adduct for chromatin structure and gene expression. Dr. Springer will conduct the enzyme assays and participate in the protein/DNA adduct studies.

D. ADDITIONAL PERSONNEL

1. Clinical Pathology

a. Supervisor--C.O. Romsos. Ms. Claudia O. Romsos is a Technical Specialist II in the Clinical Pathology Group of the Toxicology Department. She joined the staff of BNW in April 1983. She has an AA degree from Boise Junior College (1959), a BA degree from Eastern Washington State University (1986), and attended Sacred Heart Medical Center School of Medical Technology (1959-1960). She is certified by ASCP (1960) as a Medical Technologist. She has held numerous positions in the Washington State Society for Medical Technology.

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Prior to joining BNW, she worked at a local hospital and in an out-patient laboratory in the Richland area (1960-1983). She has 30 years of experience and has trained in all aspects of the clinical laboratory. She functions as the Clinical Laboratory Supervisor.

Name	Title	Areas of Responsibility	Years of Experience
DL Redetzke	Clinical Technician; MT (ASCP)	Hematology; clinical chemistry; urinalysis; & special assays	
KH Debban	Technical Specialist; MT (ASCP)	Hematology; clinical chemistry; urinalysis; & special assays	REDACTED
SL Martell	Technical Specialist; MT (ASCP)	Hematology; clinical chemistry; urinalysis; & special assays	
AE Jarrell	Technical Specialist; MT (ASCP)	Hematology; clinical chemistry; urinalysis; and special assays.	REDACTED
MM Jones	Technician	Blood collection	
CM Abraham	Technician	Blood collection	
JJ Wodtli	Technician	Blood collection	REDACTED

2. Program Administration

a. Department Administrator--P.S. Ruemmler: Ms. Patricia S. Ruemmler is the Department Administrator for the Toxicology Department. She received her BA degree from Mills College, Oakland, CA, with a major in Biology. Ms. Ruemmler is responsible for monitoring financial and contractual performance, including tracking contract modifications and updating/revising the work breakdown structure. She maintains milestone schedules and assists the PI in keeping Line Management and the Sponsor informed of potential or existing problems with those schedules. She coordinates all technical and business requests for proposals with the technical staff, BNW finance, and contracts departments. Ms. Ruemmler is responsible for supervising all the activities of the Inhalation Toxicology Program Office. These activities include records management, coordination of monthly reports, maintenance of milestone and deliverable schedules and the archiving and shipping of study data. Prior to assuming this position, she served as a QA Auditor in the GLP QA Unit of the Life Science Center where she was responsible for ensuring that projects conducted under GLP regulations for nonclinical laboratory studies were in compliance with those regulations. She has worked at BNW since 1972 and has an extensive background in many technical areas. Prior to that she was a technician in the Molecular and Cell Biology Section of the Biology and Chemistry Department for 7 years where she assisted on narcotic antagonist studies, iron chelating studies, and on numerous metabolism studies on actinide absorption in both adult and neonatal rats, dogs, guinea pigs and Hanford miniature swine.

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Name	Title	Areas of Responsibility	Years of Experience
AM Busselman	Administrative Assistant	Financial support, updating monthly budgets and cost details; maintaining labor matrices and other project management spread sheets	REDACTED
LJ Eberhart	Senior Clerk	Typing, mailings, duplicating, and general clerical support for staff	
ME Mericka	Sr. Administrative Assistant	Document processing, recordkeeping, technical editing, and report compilation	
BJ Van Zandt	Sr. Administrative Assistant	Generating milestone schedules; monitoring study events; archiving study records; records management; coordinating Monthly Report	REDACTED

3. Toxicology

a. Lead Specialist–J.T. Pierce. Ms. Judy T. Pierce is a Technical Specialist III in the Inhalation Toxicology Technical Group within the Toxicology Department. She joined Battelle in the Animal Resources Group in 1977, and was promoted to Technical Specialist in 1987. She earned her Bachelor's degree from the University of Idaho in 1977, and holds certification as a Laboratory Animal Technician through the American Association for Laboratory Animal Science. As Lead Technical Specialist, she is responsible for training and supervising other Toxicology Technical Specialists and Technicians in the Inhalation Toxicology Technical Group. Routinely, she monitors study progress, performs in-life clinical observations, and reviews in-life data printouts. She also assists in protocol, SOP, and study report preparation.

Name	Title	Areas of Responsibility	Years of Experience
DL Bove JC Blessing TM Luders	Toxicology Specialists	Supervise & train technicians; coordinate schedules; prepare SOPs & reports; perform in-life clinical observations & review in-life data	REDACTED

4. Xybion Path/Tox Computer Software

a. Assistant System Manager–J.C. Blessing. Mr. John C. Blessing is a Technical Specialist II in the Inhalation Toxicology Technical Group within the Toxicology Department. He joined BNW in the Animal Resources Group in 1986 and was promoted to Technical Specialist in 1989. He earned his BA degree from Washington State University in 1985 and holds certification as a Laboratory Animal Technician through the American Association for Laboratory Animal Science.

Mr. Blessing has 7 years of experience with the Xybion Path/Tox computer software and has participated in a variety of capacities in data collection. He is currently the designated Assistant System Manager. As the Assistant System Manager, he assigns operator privileges, trains and assists technicians, toxicologists, and pathologists in their use of the software, and assumes overall responsibility for records maintenance, general diagnostics and remediation, software validation, and correspondence with Xybion Medical Systems.

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5. Animal Resources

a. Supervisor--A.P. Wagner. The Animal Resources Group is lead by Ms. Alison P. Wagner. Ms. Wagner has an Associate of Science in Veterinary Technology degree from Pierce College. She has 6 years of experience in laboratory animal care at BNW. Ms. Wagner has been LSL-II Animal Resources Supervisor since August 1992 and prior to that, was Assistant Supervisor for the facility for 2 years. Before that, she was an Animal Resources Specialist. Her duties include supervising and training technicians, coordinating schedules, directing facility maintenance and animal care, and preparing SOP's and reports.

Ms. Wagner supervises the activities of 24 technicians. Six of these people have 17 or more years of experience in laboratory animal care at BNW. Seven of these individuals are American Association of Laboratory Animal Science (AALAS)-certified Laboratory Animal Technicians; two are AALAS-certified Assistant Laboratory Animal Technicians.

The Animal Resources Staff provide for the daily care and routine daily observation of the animals. They also interact closely with the toxicology staff and the exposure staff. They assist with the collection of clinical observation and weight data; tattoo the animals; maintain sanitation throughout the facility; and wash and changeout racks, cages and related equipment.

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Name	Title	Areas of Responsibility	Years of Experience
VL Dedmond	Senior Animal Technician	Daily check for moribundity & mortality; daily animal husbandry procedures; animal facility sanitation; clinical observations; body weights	REDACTED
GL Miller	Senior Animal Technician		
PA Pierce	Senior Animal Technician		
DC Snyder	Senior Animal Technician		
FL Wallace	Senior Animal Technician		
CM Abraham	Animal Technician		REDACTED
JE Caldwell	Animal Technician		
SW Chase	Animal Technician		
MS Cockburn	Animal Technician		
JT Dvorak	Animal Technician		
SF Harris	Animal Technician		
MM Jones	Animal Technician		REDACTED
DR Kimsey	Animal Technician		
JP Rogers	Animal Technician		
MA Skogley	Animal Technician		
SL Stewart	Animal Technician		
SA Webb	Animal Technician		
JJ Wodtli	Animal Technician		
SA Breier	Animal Technician (new hire)		REDACTED
EO Cox	Animal Technician (new hire)		
B Gillard	Animal Technician (new hire)		
CR Helland	Animal Technician (new hire)		
PM Kopf	Animal Technician (new hire)		REDACTED
JW Short	Animal Technician (new hire)		

6. Veterinary Services

a. Veterinary Service Specialist--K.H. Debban. The animal health program is directed by Dr. S.E. Rowe, a Diplomate of the American College of Laboratory Animal Medicine. Dr. Rowe is supported in the laboratory by Ms. Kathryn H. Debban, an ASCP-certified Medical Technologist with 24 years of experience in medical technology. She has worked as a medical technologist for BNW since 1975 and has worked in the veterinary services diagnostic laboratory since January 1991.

Also available on a call-in basis is Ms. Kimberly G. Pauly. Ms. Pauly is also an ASCP-certified Medical Technologist. She has 6 years of experience in medical technology.

Both Ms. Debban and Ms. Pauly are experienced and qualified to do serological testing for viral antibodies, culture and identification of bacterial pathogens, and water culture and monitoring techniques. They are involved with nearly all aspects of the health care program including preexposure health screening, testing sera for viral antibodies, drinking water quality monitoring, and report preparation. The veterinary services laboratory participates in the Rockefeller University QA program in serology and bacteriology for laboratory animal diagnostic laboratories.

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7. Pathology

a. Necropsy and Histology Supervisor--K.M. Gideon. Ms. Katherine M. Gideon is a Senior Technical Specialist in the Pathology Group of the Toxicology Department. She joined the staff of BNW in October 1980. She has an AA degree from Spokane Falls Community College (1976), attended Sacred Heart Medical Center School of Histologic Technique (1976-1977), and was certified by the American Society of Clinical Pathologists (1977) in the field of histology. She is the president of the Washington State Histology Society and has presented several posters, workshops and lectures at state and national meetings in the field of veterinary histology and techniques.

Prior to joining BNW, she worked at Sacred Heart Medical Center, Spokane, WA (1976-1977), Pathology Associates, Spokane, WA (1977), and Washington Animal Disease Diagnostic Laboratory, Washington State University - Pullman, WA (1977-1980). She has 16 years of experience in histology and has trained in all aspects of technical procedures of histology and necropsy. She functions as the Histology Laboratory Supervisor in coordinating efforts with histology/necropsy technicians in performing the duties of necropsy, trimming, slide preparation, and repository shipping.

Name	Title	Areas of Responsibility	Years of Experience
SM Baze	Necropsy Supervisor	Necropsy; IANR data entry & review	
TA Breier	Histology/Necropsy Tech.	Histology/necropsy technicians are cross-trained and able to perform duties in both areas (histology & necropsy) depending on the situation.	REDACTED
AL Reed	Histology/Necropsy Tech.		
TS Bisson	Histology/Necropsy Tech.		
RF Flores	Histology/Necropsy Tech. HT (ASCP)		
HW Hill			
MD Littleton	Histology/Necropsy Tech.		REDACTED
BG Moore	Histology/Necropsy Tech.		
MA Pope	Histology/Necropsy Tech. HT (ASCP)		
SJ Schmaljohn			
JD Stearns	Histology/Necropsy Tech.		
KG Studniski	Histology/Necropsy Tech.		
RM Fairweather	Histology/Necropsy Tech.		
LA Wisse	Histology/Necropsy Tech.		REDACTED
NS Marvin	Histology/Necropsy Tech.		
JL Darnell	Histology/Necropsy Tech.		
SV Hall	Histology/Necropsy Tech.		
WA Holstein	Histology/Necropsy Tech.		
DA Millman	Histology/Necropsy Tech.		
MR Moore	Histology/Necropsy Tech.		
K Goodey	Histology/Necropsy Tech.		REDACTED
	Histology/Necropsy Tech.		
	Histology/Necropsy Tech.		
	Histology/Necropsy Tech.		
	Histology/Necropsy Tech.		

8. Chemistry

a. Chemistry Specialists. The Chemistry Specialists are responsible for the setup, optimization, and maintenance of a wide variety of chromatographic and spectrophotometric instrumentation. They perform first-echelon data review as well as production of Standard Operation Procedures for routine operations. They train, supervise, and schedule the activities of the Chemistry Technicians in the operation,

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maintenance, and calibration of the exposure monitoring systems and in the performance of special studies such as chemical stability studies, toxicokinetic or bioanalytical studies. The Chemistry Specialists are responsible for modification of instrumentation to perform special analytical operations (typically on-line exposure monitoring). The Chemistry Specialists coordinate routine and nonroutine tasks with other technical groups. They are responsible for assuring that Good Laboratory Practices are followed in the conduct of data collection. The Chemistry Specialists are responsible for the compilation of reports and in preparing technical input for proposals and scientific publications.

Name	Specific, Pertinent Study Experience	Years of Experience
DJ Anderson	Prechronic studies of gluteraldehyde. Prechronic and chronic studies of Ozone. Ozone on-line monitoring by UV spectrophotometer. Experienced with GC, GC/MS, ICP-AES, HPLC instrumentation and methods development. Prechronic and chronic studies of 7 vapor studies. Prechronic and chronic studies of 7 pharmaceutical studies with GC and HPLC support. Assisted with 2-butoxyethanol metabolite studies.	REDACTED
BK Hayden	Prechronic and subchronic studies on mainstream tobacco smoke. Prechronic humectant studies in support of tobacco research. Liquid aerosol studies in support of tobacco research. Sponsor submitted plasma samples for determination of nicotine and cotinine in support of tobacco research. Experience includes: exposure atmosphere assessment for nicotine, humectants, and liquid aerosols; chromatographic determination of percent carboxythemoglobin from hamster blood; chromatographic determination of nicotine and cotinine in animal blood. Chemical constituent measurements for solvent refined coal and shale oil studies. Experience includes: chemical class fractionation; chromatographic identification and quantitation; physical properties measurements. Prechronic, subchronic, and chronic studies of 9 organic vapor studies. Prechronic study for GaAs. Prechronic, subchronic, and chronic studies of 9 pharmaceutical studies. Experienced with GC/MS, GC, LC, ICP-MS, instrumentation and methods development. Bioanalytical measurements of parent and metabolite compounds in blood and urine from animals exposed to styrene, acetone, acetonitrile, 1-nitropyrene, and furfuryl alcohol.	
RE Hauber	Prechronic, subchronic and chronic studies of 5 inorganic dust studies. Chronic studies with biological assays for MoO_3, GaAs and Ga_2O_3 by ICP-AES and GFAAS. Experienced with ICP-AES, GFAAS/AAS, and GC instrumentation and methods development. Prechronic and chronic studies of 9 pharmaceutical studies with HPLC support. Assisted in 6 vapor studies.	REDACTED
AD Marcy	Prechronic studies of Gallium Arsenide. ICP-MS support for bioanalytical assays of lead. Experienced with IC, FAAS, ICP-AES, ICP-MS instrumentation and methods development.	
KH Mellinger	Prechronic and subchronic studies on mainstream tobacco smoke. Prechronic humectant studies in support of tobacco research. Experience includes: exposure atmosphere assessment for nicotine. Prechronic, subchronic, and chronic studies of 6 organic vapor studies. Prechronic, subchronic, and chronic studies for 5 inorganic dust studies. Experienced with ICP-AES, GFAAS and FAAS instrumentation and methods development. Also experienced with IR, GC, UV-VIS and titrimetric methods. Bioanalytical measurements of parent compounds in blood, urine and tissues from animals exposed to GaAs, MoO_3, Ga_2O_3, CdO, and InP. Radioanalytical measurements in support of radiolabeled aerosol drug study. Experienced with Sample Oxidizer and Scintillation Counter.	
RJ Moore	Experience in operation and applications development for the following instrumentation: GC, GC/MS, HPLC, and LSC. Experience in operation of the following instrumentation: ICP-AES, GFAAS and FAAS. Lead specialist for 2 chronic vapor studies. Assisted in 10 chronic vapor and 5 chronic dust studies. Further experience includes: analytical methods development for pharmaceuticals and pesticides, instrumentation modifications for vapor analyses, and assistance in metabolite studies.	REDACTED

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b. Chemistry Technicians. The Chemistry Technicians perform routine sample preparation and analysis following written analytical procedures. They perform data workup and routine report generation. They assist in the implementation of new analytical methods, method development and the development of procedures for special studies such as chemical stability studies, toxicokinetic, and bioanalytical studies. The Chemistry Technicians are involved in the maintenance of analytical instrumentation, maintenance of test material inventory, and procurement of necessary supplies. They are responsible for routine recordkeeping according to Good Laboratory Practices. Due to a program of aggressive cross-training, each chemistry technician is capable of performing almost every technician task. As a result, technicians are not normally assigned primary responsibility for a particular study.

Name	Specific, Pertinent Study Experience	Years of Experience
SA Barraclough	Assisted with 14 day and 3 month mainstream smoke studies in rodents. Assisted with glutaraldehyde prechronic studies. Assisted in over 12 prechronic and chronic vapor studies.	REDACTED
SL Crowner	Assisted with isoprene and isobutene prechronic and chronic studies. Assisted in 6 prechronic and chronic vapor studies. Assisted in prechronic radiologic pharmaceutical studies.	
HA Droppo	Assisted with prechronic radiologic pharmaceutical studies. Assisted in 4 prechronic and chronic vapor and dust studies.	REDACTED
BD Macisaac	Assisted with 8 prechronic and chronic pharmaceutical studies. Assisted in 2 prechronic vapor studies.	
LL Reed	Assisted with isoprene and isobutene prechronic and chronic studies. Assisted with 4 prechronic and chronic pharmaceutical studies.	
KK Weitz	Assisted with isoprene and isobutene prechronic and chronic studies. Assisted with furfuryl alcohol and 2-butoxyethanol urinary metabolite studies. Assisted in 4 vapor and dust studies. Assisted in 3 prechronic and chronic pharmaceutical studies.	REDACTED

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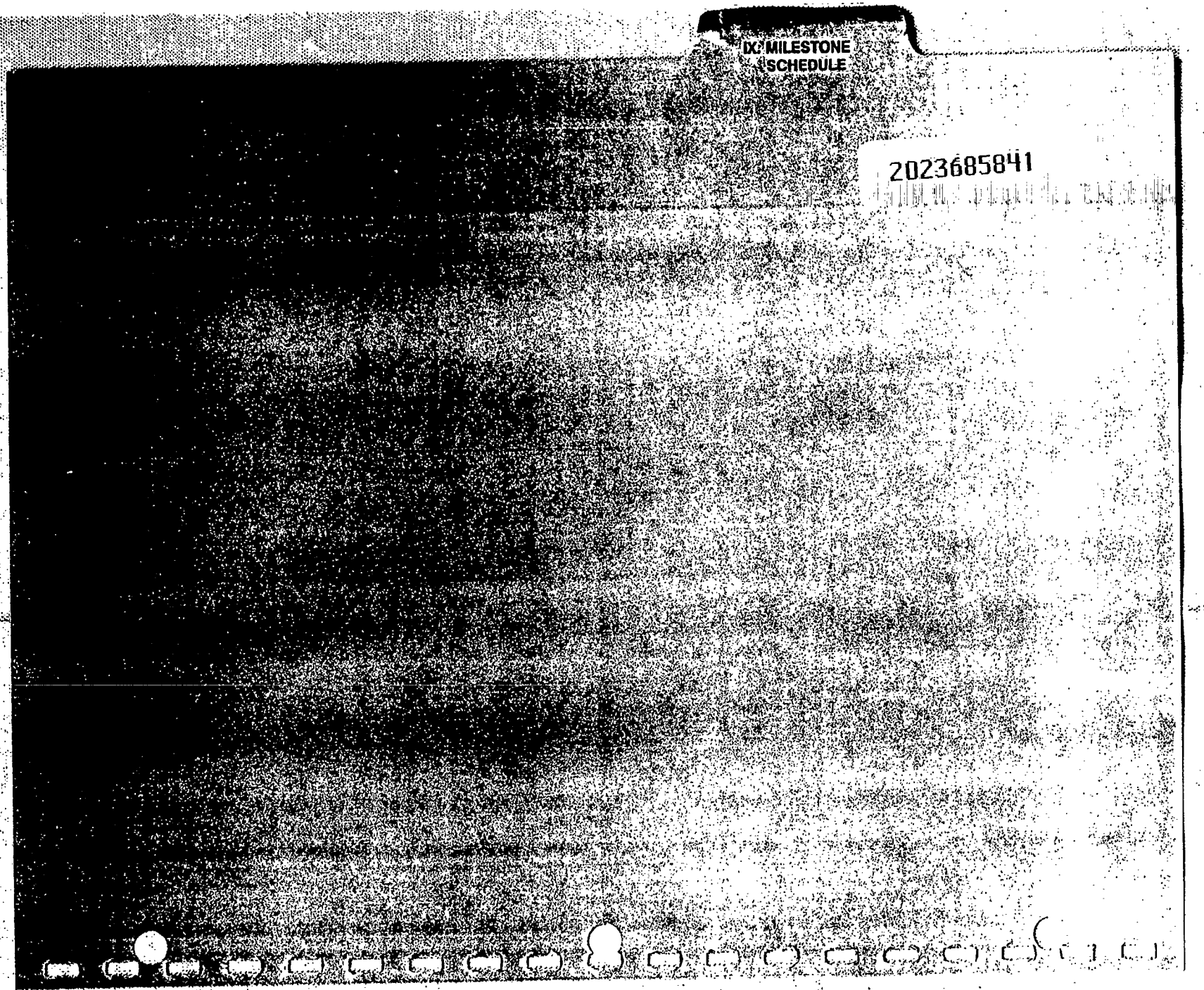

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IX. MILESTONE SCHEDULE

For purposes of scheduling and cost estimates, it has been assumed that the contract will be awarded January 1, 1994.

Milestones have been set to provide for adequate staffing and scheduling of the exposure rooms, exposure starts, and necropsies (shown on pp. IX-2 and IX-3). The time estimates and cost estimates are predicated on the following assumptions: ability of animal suppliers to deliver, on time, the required number of mice; on-time arrival of the test article; and final experimental design specifications and toxicologic parameters to be determined do not differ significantly from those in this technical proposal.

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PROVIDE INHALATION TOXICITY STUDIES OF CLIENTS PROPRIETARY PRODUCT
MILESTONE SCHEDULE

CONTRACT AWARD	12/31/93
CONTRACT START	1/1/94
Systems design and parts procurement	Jan-1994 (4 months)
Pilot Study	Mar-1994 (3 months)
Construction generation, distribution, monitoring and exposure systems	May-1994 (6 months)
START PHASE 1	Nov-1994
Sponsor Review/Approval Prestart Reports, order animals	May-1995
90-DAY - For-Nose Only	
START EXPOSURE	Aug-1995
TERMINAL SACRIFICES	Oct-1995
Submit Reports to sponsor	Jul-1996
Review Reports and approve carcinogenicity study	Aug-1996 (6 months)
CARCINOGENIC - For-Nose Only	
START EXPOSURE	Mar-1997
Respiratory Minute Volume	
6 Months	Sep-1997
12 Months	Mar-1998
18 Months	Sep-1998
23 Months	Feb-1999
Carboxyhemoglobin of the blood	
6 Months	Sep-1997
12 Months	Mar-1998
18 Months	Sep-1998
23 Months	Feb-1999
Nicotine and nicotine metabolites in the urine	
6 Months	Sep-1997
12 Months	Mar-1998
18 Months	Sep-1998
23 Months	Feb-1999
Particle clearance from the lungs	2 times/study
Lung Burden	
15 Months	Jun-1998
18 Months	Sep-1998
Differential blood cell count	
Terminal Sac	Mar-1999

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PROVIDE INHALATION TOXICITY STUDIES OF CLIENTS PROPRIETARY PRODUCT
MILESTONE SCHEDULE

CARCINOGENIC - For-Nose Only (continued)	
INTERIM REPORTS	
12 Months	Mar-1998
18 Months	Sep-1998
TERMINAL SACRIFICES	Mar-1999
REPORT DUE	Feb-2000
Manuscripts for publication prepared and approved by sponsor and submitted to journals	Aug-2000
Archive materials submitted to sponsor	Dec-2000

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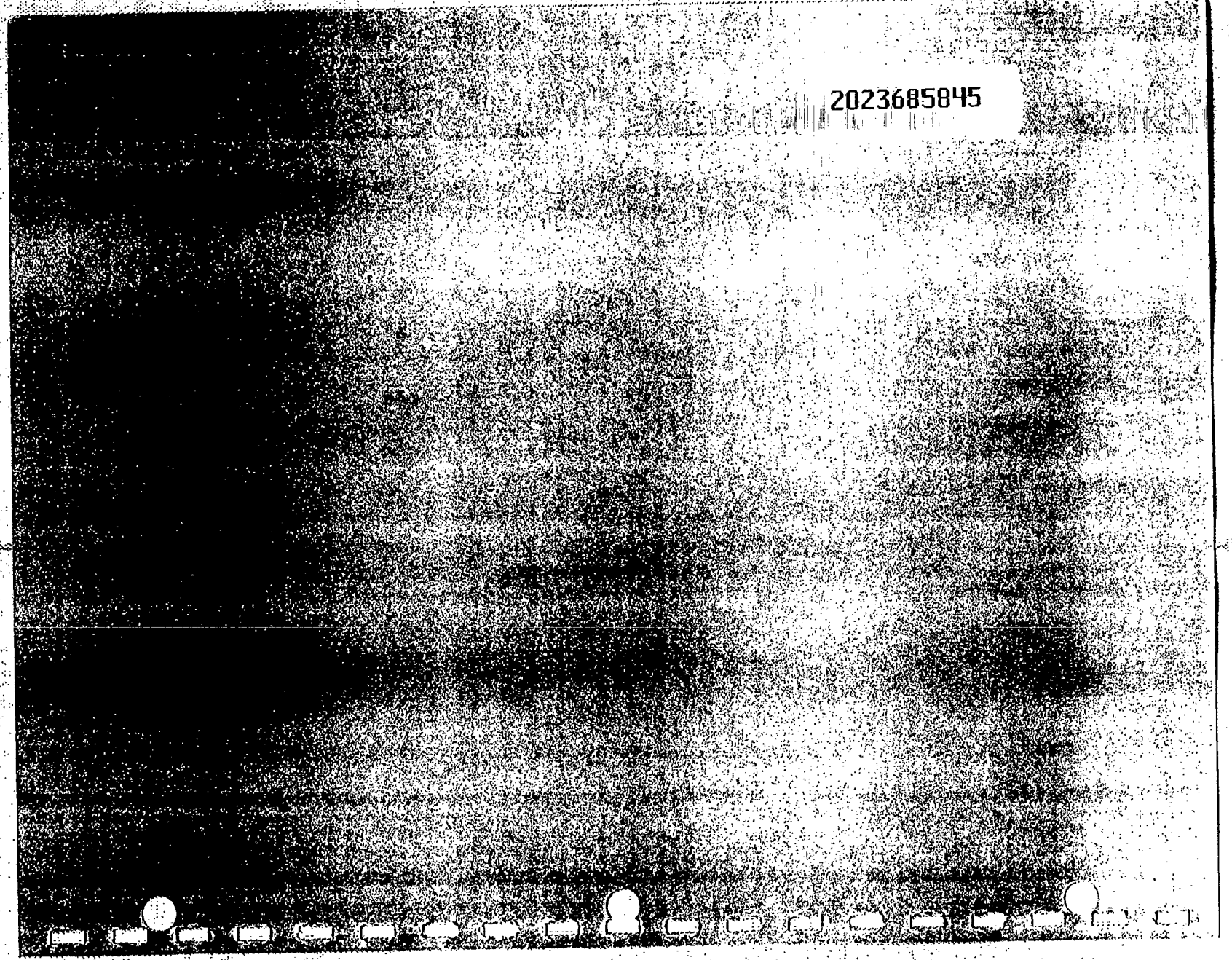

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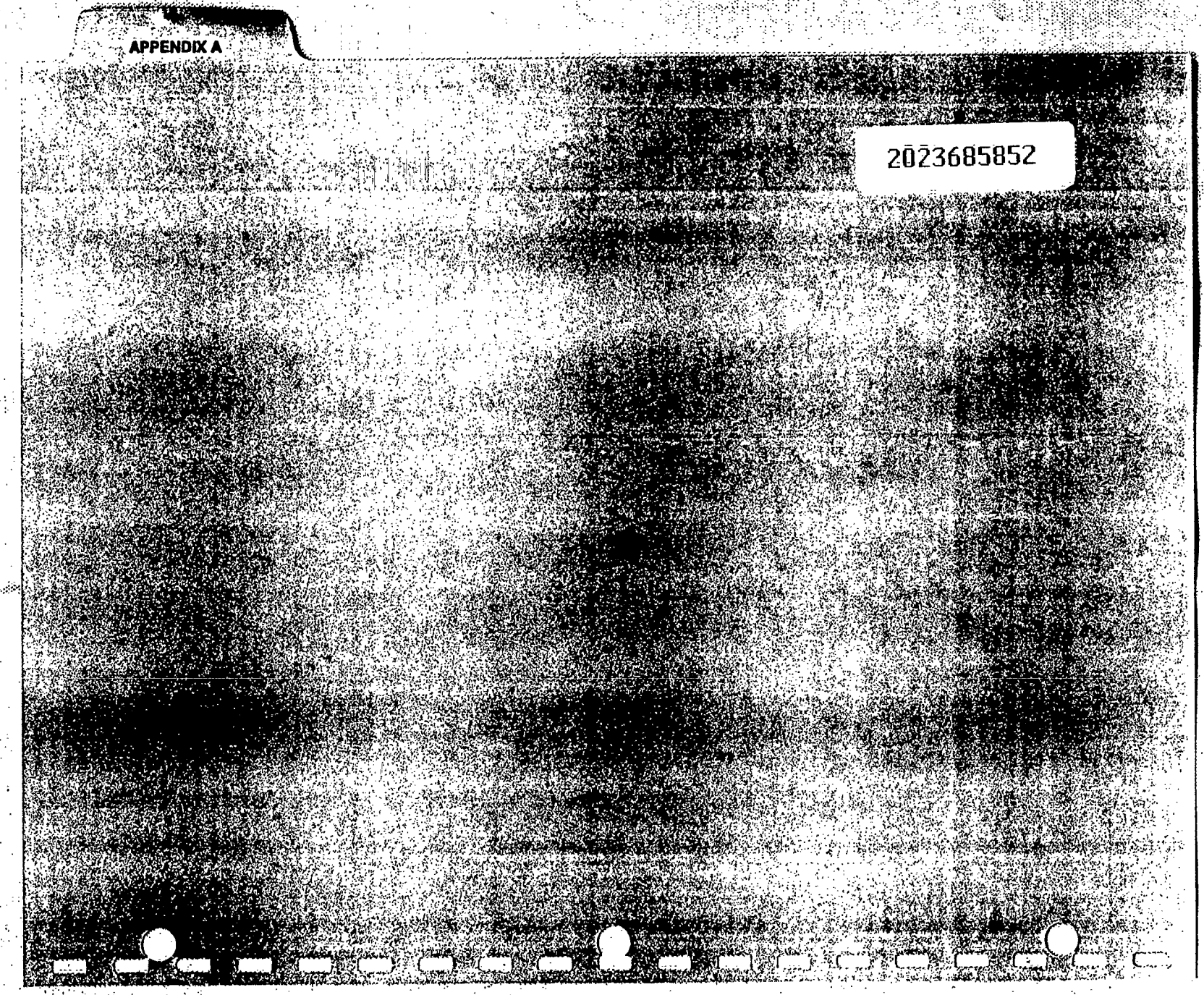

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APPENDIX A

DRAFT PROTOCOLS

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TWO-YEAR CARCINOGENICITY NOSE-ONLY INHALATION STUDY OF ROOM AGED CIGARETTE SIDESTREAM SMOKE AND DIESEL ENGINE EXHAUST IN MICE

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TWO-YEAR CARCINOGENICITY NOSE-ONLY INHALATION STUDY OF ROOM AGED CIGARETTE SIDESTREAM SMOKE AND DIESEL ENGINE EXHAUST IN MICE

SPONSOR:
Philip Morris
Research Center
P.O. Box 26583
Richmond, Virginia 23261

Protocol Date: _____

CONTRACT LABORATORY:
Battelle, Pacific Northwest Laboratories (BNW)
Life Sciences Laboratory (LSL-II Building)
P.O. Box 999
Richland, Washington 99352

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I. STUDY TITLE

Two-Year Carcinogenicity Nose-Only Inhalation Study of Room Aged Cigarette Sidestream Smoke and Diesel Engine Exhaust in Mice

II. PROJECT OBJECTIVE

The objective of this study is to determine the chronic carcinogenic potential of RASS administered by nose-only inhalation to male and female mice 6 hours daily, 5 days/week for two years. A diesel engine exhaust (DEE) exposure group will be included as a positive control. Non-neoplastic and neoplastic lesions particularly in the respiratory tract will be of major interest. Systemic effects (e.g., changes in body and organ weights, signs of intoxication) will also be determined. Effects will be compared to those seen in the sham-exposed (filtered air) negative and DEE-exposed positive control groups. Biomonitoring will be performed on RASS- and DEE-exposed rats.

III. STUDY SCHEDULE

A. Mice Arrive - week of

B. Start of exposure -

C. Last day of exposure -

IV. PROJECT NUMBER

V. PROTOCOL DATE

VI. SPONSOR

Philip Morris
Research Center
P.O. Box 26583
Richmond, Virginia 23261

VII. SPONSOR'S REPRESENTATIVE

George J. Patskan, PhD

VIII. TESTING FACILITY

Battelle, Pacific Northwest Laboratories (BNW)
Toxicology Department K4-10
P.O. Box 999
Richland, Washington 99352

IX. STUDY DIRECTOR AND PRINCIPAL INVESTIGATOR

Earl W. Morgan, DVM, ACVPM, DABT

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X. TEST ARTICLE DESCRIPTION

A. Identification

1. Room Aged Sidestream Smoke (RASS):

RASS will be obtained by aging sidestream smoke (SS) for 0.5 hours (mean age) in a room. SS will be generated from the standard reference filter cigarette, 1R4F, smoked in basic conformity with ISO standards 3402, 4387, and 3308 (1991). The cigarettes will be smoked by a 30-port smoking machine (CH Technologies, Westwood, NJ).

B. Lot Number

C. How Supplied

D. Storage Conditions

Cigarettes will be stored in a freezer at -16 ± 2°C. They will be preconditioned for at least 48 hours at room conditions (22 ± 2°C and 60 ± 10% RH) prior to smoking. Distribution and handling during the course of the study will be conducted in such a manner that proper identification will be maintained and contamination avoided.

E. Source

Philip Morris

XI. POSITIVE CONTROL DESCRIPTION

A. Diesel Engine Exhaust

The DEE will be generated from a light-duty pickup truck engine, mounted on a shock mount engine stand attached to a dynamometer system. The dynamometer will be programmed to simulate the EPA Urban Dynamometer Driving Schedule (40CFR Ch. 1, Pt. 86, App I).

B. Storage Condition

Diesel fuel will be stored in tank external to the building with a small day tank in the generator room.

C. Source

XII. TEST ATMOSPHERE

On-line monitoring of the mass concentration of RASS and DEE will be accomplished by two Tapered Element Oscillating Microbalance Mass Monitors (TEOM Model 1400; Ruppprect & Patashnick Co., Inc., Albany, NY). The TOEMs will sample RASS and DEE aerosols from a representative exposure port in the exposure unit. Exposure concentrations will be controlled to within ±20 % of target concentration by adjustment of the exposure unit dilution airflow. Mass concentration will be monitored in one of each pair of exposure units at a time.

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A real time aerosol monitor (RAM; MIE, Inc., Bedford, MA) will be used to monitor the concentration of RASS as it enters the distribution line from the aging room. Aerosol concentrations will be monitored with the RAM twice per hour over the duration of the daily generation period. The relative response of the RAM to the aerosol concentration (volts per unit concentration) will be determined prior to the start of exposure. RAM response will be evaluated by comparing the measured RAM voltage to the aerosol concentration of RASS determined from independent filter samples.

The total particulate mass (TPM) of both RASS and DEE in the test atmosphere during the exposure will be determined from duplicate Cambridge filter samples from both the distribution line at the exit from the aging room and two exposure ports from each exposure unit collected over the duration of the generation period. These filters will be analyzed gravimetrically and/or by UV analysis. Filter sample extracts will be analyzed as soon as feasible following collection and preparation.

The buildup and decay of the test article concentration in the exposure unit will be determined prior to start of the study. The time to buildup to 90% of the target concentration (T_{90}) and the time to decay to 10% of the target concentration (T_{10}) will be determined.

The particle size distribution of the aerosol in the exposure unit will be measured prior to the start of exposure and once a month during the exposure phase. Median diameters and geometric standard deviations (GSD) will be reported.

Uniformity of the concentration and particle size distribution of the test article in the nose-only exposure unit will be determined during the prestart phase of the study. Uniformity of test article concentration will be demonstrated by monitoring one port on each level with the TEOM or RAM. Uniformity of particle size distribution will be demonstrated by samples taken at the top, middle and bottom levels of the exposure unit.

XIII. INHALATION EXPOSURE SYSTEM

A. Exposure Room

Rooms 523 and 531, LSL-II Building

B. Exposure Unit

Cannon flow-past nose-only exposure units. Each unit has 104 exposure ports, 90 of which will be used for rat exposures. The remaining ports will be used for sampling or closed off. Each exposure unit will be contained in a rigid clear plastic cabinet to assure no contamination of the room by the test article aerosol. Airflow through the hood will be maintained at approximately 15 cfm to assure proper cooling of the mice in the restraint tubes. The exposure unit can be rotated so that all mice may be easily observed during exposure.

C. Exposure Generator

RASS will be obtained by aging sidestream smoke (SS) for 0.5 hours (mean age) in a 30 m^3 room. SS will be generated from the standard reference filter cigarette, 1R4F, smoked by a 30-port smoking machine (CH Technologies, Westwood, NJ) in basic conformity with ISO standards 3402, 4387, and 3308 (1991). Mainstream smoke from the smoking machine will be routed through a filter system to remove the majority of the particulate material prior to being routed to the building exhaust. A stainless steel cone will be fitted over the smoking machine to extract the SS which will be mixed with conditioned dilution air (22 ± 2°C and 60 ± 10% RH) before being injected into a smoke aging room by a flow-controlled fan. The

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entire flow from the aging room will be delivered to the exposure rooms via a stainless steel delivery tube. The required flow of RASS will be siphoned from this delivery line by a pressurized-air-controlled ejector tube, mixed with conditioned air to achieve the proper concentration, and injected into the nose only exposure unit. Exhaust from the exposure units will be routed through a filter system to remove the majority of the particulate material prior to being routed to the building exhaust.

DEE will be generated from a light-duty pickup truck engine, mounted on a shock mount engine stand attached to a dynamometer system. The dynamometer will be programmed to simulate the EPA Urban Dynamometer Driving Schedule (40CFR Ch. 1, Pt. 86, App I). A constant flow of DEE from the engine will be delivered from a plenum downstream of the engine muffler to the exposure rooms through a heated stainless-steel deliver line. The required flow of DEE will be siphoned from this delivery line by a pressurized-air-controlled ejector tube, mixed with conditioned air to achieve the proper concentration, and injected into the nose only exposure unit. All DEE exhaust will be routed to the building exhaust.

XIV. TEST SYSTEM SPECIFICATIONS

Species:	Mus musculus
Strain::	TBD (VAF)
Source::	Charles River Laboratories Raleigh, NC
Age at Arrival::	4-5 weeks
Age at Study Start::	6-7 weeks
Total Number of Animals:	475 males; 475 females
Exposure Tube Acclimation Period:	~5 days
Identification:	Tail tattoo

XV. REASON FOR SELECTION

The ____ mouse was selected for this study on the basis of data available in the literature and a 13-week pilot study.

XVI. RANDOMIZATION

The mice which adapt to the exposure tubes will be randomly assigned to exposure groups by using body weight as a blocking variable to ensure that there are no statistically significant differences in initial group mean body weights. The weight distribution range (by sex) of the mice selected for the study will be as narrow as possible. A sufficient quantity of mice will be ordered and adapted to the exposure restraint tubes to ensure that no mouse will be selected whose body weight exceeds ±20% of the mean (by sex) at the start of the study. The Xybion Path/Tox System (Xybion Medical Systems Corporation, Cedar Knolls, NJ) will be used for randomization.

XVII. HOUSING AND MAINTENANCE

Mice shipping crates will be examined upon arrival for evidence of conditions likely to permit exposure to pathogens (soiled, wet, or otherwise damaged). The uncrating will be conducted at the door of the animal room. While being removed from the crates, the mice will be examined for evidence of shipping stress.

The mice will be acclimated in Room ____ of the LSL-II Building for 2 weeks prior to the start of exposure. Mice will remain on quarantine status until health screen procedures are completed, approximately 3 weeks after their arrival.

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Throughout the study, cage space will meet the requirements stated in the 85-23 (1985) NIH "Guide for Care and Use of Laboratory Animals". The BNW animal care and use program, including the facility used for this study, is accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

The mice will be housed approximately 5 mice per cage in hanging wire cages prior to identification and randomization at which time they will be housed individually in stainless steel wire cage units on stainless steel racks. Mice will be individually housed for the remainder of the study.

Room temperatures during the acclimatization and study will be maintained at 75 ± 2°F and relative humidities at 55 ± 15%. Measurements will be recorded at ~1 hour intervals.

Twelve hours light and twelve hours dark will be maintained with light starting at 0600.

Within 48 hours of arrival 5 mice/sex will be examined by gross necropsy, histopathology, and serological testing for evidence of disease and the presence of potentially pathogenic organisms. Sera will be tested for antibodies to Sendai virus, mouse hepatitis virus, GDVII virus, minute virus of mice, pneumonia virus of mice (PVM) and *Mycoplasma pulmonis*. Mice will be released for study if the health screen doesn't provide evidence of pathogens or disease. The clinical veterinarian will make a visual inspection of the mice to be used in the study just prior to their release for the study. Five mice per sex that were not used for the exposures will be reevaluated for evidence of disease 18-21 days after arrival at BNW.

Sentinel mice (15M,15F) will be selected from the extra mice prior to the start of exposures. Serological evaluations will be performed on 5/sex at 6, 12, and 18 months. Serological evaluations will be performed on 5/sex at the time of terminal sacrifice from the filtered air control group of mice.

During the acclimation period, approximately 7-10 days prior to the start of exposure, mice will be placed in nose-only exposure restraint tubes for increasing time up to 6 hours daily to allow them to adapt to confinement in the tubes. The mice which adapt to the restraint tubes will then be weighed, randomized, assigned to exposure concentration groups and identified individually with a unique number tail tattoo approximately two days before the exposure period.

Mice not used for the study will be euthanized by inhalation of 70% CO_2 one week after the start of the study. The disposition of these mice will be recorded on the Animal Disposition Record and retained in the study files.

Mice which die during the first week of the study will be replaced from the pool of unused mice.

XVIII. DIET AND WATER

NIH-07 Open Formula diet will be provided *ad libitum* except during the inhalation exposures when mice are in restraint tubes.

This diet is analyzed for contaminants and some nutrient components; these analyses are reviewed for acceptability using established standards prior to use of the food. There are no known contaminants in the diet which could be expected to alter the outcome of the study.

Fresh softened water (ion exchange softener, Illinois Water Treatment Company, Model 2R-2240, Rockford, IL) will be supplied *ad libitum* except during exposures. An automatic watering system (Edstrom Industries, Waterford, WI) will be used during the quarantine/acclimatization period and throughout the study. Water bottles will also be provided for the first three days after arrival.

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Representative samples of animal drinking water from the LSL-II facility will be analyzed for contaminants at least once per year. There are no known contaminants in the drinking water which could be expected to alter the outcome of the study. The hardness of the water will be checked approximately once every week. Records will be retained in the LSL-II Building Engineer's office.

XIX. ENVIRONMENTAL MONITORING

A. Air Supply

Room and exposure supply air will be HEPA-filtered. Both room and exposure exhaust air will pass through a series of two HEPA filters.

B. Temperatures

Temperatures in each exposure unit cabinet and in the exposure air stream at a nose port will be monitored at ~2-minute intervals and recorded at ~60 minute intervals during the daily exposure period. If the temperature is not within the acceptable range 75 ± 4°F, the system will alarm. Daily means for the exposure cabinet and the exposure atmosphere will be maintained within 75 ± 4°F.

Temperatures in the animal room will be monitored at ~2-minute intervals and recorded once every hour. If the temperature is not within the acceptable range 75 ± 2°F, the system will alarm. The acceptable range for individual animal room measurements and the daily mean will be 75 ± 2°F.

C. Relative Humidity

Relative humidity will be monitored at a nose-port in each exposure unit and recorded at ~60 minute intervals during the exposure period. If the relative humidity is not within the acceptable range 55 ± 15%, the system will alarm. Daily means for the exposure unit will be maintained within 55 ± 15%.

Animal room humidity will be monitored and recorded approximately once every hour. If the relative humidity is not within the acceptable range 55 ± 15%, the system will alarm. The acceptable daily mean is 55 ± 15% relative humidity.

D. Airflow

Airflow will be monitored at an inlet and exhaust orifices using a calibrated photohelic pressure gauge. Both flows will be recorded once per hour during the exposure period. The acceptable minimum inlet flow is 250 ml/min per occupied exposure port.

Measurements out of specified limits will be recorded and result in an alarm so that the operator can take appropriate actions.

XX. EFFLUENT TREATMENT

Exposure unit exhaust will be passed through a series of two HEPA filters and a catalytic converter to remove all test article. The concentration of test article in the building exhaust will be measured once during the study to determine the efficiency of the effluent treatment system.

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XXI. EXPERIMENTAL DESIGN

A. Route of Administration and Reason for Selection

The test articles will be administered by nose-only inhalation at concentrations of 1, 3 and 10 mg/m^3 of RASS and ____ mg/m3 of DEE. A filtered air control group will be included. The concentration of RASS and the route of administration used in this study were selected on the basis of route of human exposure and to provide target particle concentrations which exceed those determined for extreme human exposure to environmental tobacco smoke by a factor of approximately 10. The DEE concentrations were selected on the basis of data in the literature which indicate that this concentrations should result in a measurable carcinogenic response in the mouse and a comparable total particulate matter content to the RASS exposure.

B. Experimental Design

Group Number	Exposure Concentration (mg/m^3)	Core Study Mice	Lung Burden and Lung Clearance[a]	CoHb[b]	Total
1	0	60M 60F	18M 18F	12M 12F	90M 90F
2	RASS (1)	60M 60F	18M 18F	12M 12F	90M 90F
3	RASS (3)	60M 60F	18M 18F	12M 12F	90M 90F
4	RASS (10)	60M 60F	18M 18F	12M 12F	90M 90F
5	DEE (TBD)	60M 60F	18M 18F	12M 12F	90M 90F
Sentinels[d]	0	15M 15F	– –	– –	15M 15F

[a]Four mice/sex will be sacrificed at 4 timepoints. Two additional mice/sex/group are included to be used if needed.

[b]Five mice/sex will be bled at 6, 12, and 18 months. Mice will be allowed to survive at 6 and 12 months. Carboxyhemoglobin will also be determined from 5 mice/sex/group from the core study at terminal sacrifice.

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C. Animal Identification

Group Number	Exposure Concentration (mg/m^3)	Core Study Mice[a]	Lung Burden and Lung Clearance[a]	CoHb
1	0	1-60 (M) 101-120 (F)	61-78 (M) 161-178 (F)	79-90 (M) 179-190 (F)
2	RASS (1)	201-220 (M) 301-320 (F)	261-278 (M) 361-378 (F)	279-290 (M) 379-390 (F)
3	RASS (3)	401-420 (M) 501-520 (F)	461-478 (M) 561-578 (F)	479-490 (M) 579-590 (F)
4	RASS (10)	601-620 (M) 701-720 (F)	661-678 (M) 761-778 (F)	679-690 (M) 779-790 (F)
5	DEE (TBD)	801-820 (M) 901-920 (F)	861-878 (M) 961-978 (F)	879-890 (M) 979-990 (F)
Sentinels	0	SM1-SM15 SF1-SF15	– –	– –

D. Route of Administration

Nose-only Inhalation

E. Frequency/Duration

Six hours/day plus T_{90}; 5 days/week; up to 104 weeks; excluding weekends and holidays

F. Daily Observations

Twice daily for moribundity and mortality on all surviving mice.

G. Clinical Signs

Weekly for all surviving core study mice and prior to unscheduled sacrifice for moribund or humane sacrifice mice.

H. Body Weights

Weekly for the first 13 weeks, and at 4-week intervals thereafter (every 2 weeks during the last 13 weeks if needed)on all surviving core study mice.

I. Urine Nicotine/Cotinine

Six/sex, selected at random from the core study animals, from the RASS treated and filtered air control groups at 6, 12, 18, and 24 months. Mice will be placed in metabolism cages for a 16 hour urine collection.

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J. Carboxyhemoglobin

Five/sex/group from designated animals at 6, 12, and 18 months and 5/sex/group selected at random from the core study animals at terminal sacrifice. Mice will be removed from the exposure chamber within one hour before the end of the daily exposure period and bled from the supra-orbital sinus.

K. Clinical Pathology Evaluations

Fifteen mice/sex/group from the core study mice, prior to terminal sacrifice, will be anesthetized with ~70% CO_2 and blood samples will be collected from the retra-orbital sinus into tubes containing potassium EDTA for a complete blood count. At terminal necropsy the remaining mice and all moribund sacrifice mice will have a slide prepared for a differential leukocyte count. The slides will only be evaluated if abnormalities are detected in the CBCs.

L. Lung Clearance

During the 15th month 18 mice/sex/group will be exposed to 10 mg TiO_2/m^3 for 5 consecutive days. Lung burdens of TiO_2 will be determined using ICP-AES techniques, on 4 animals/group at time points to be determined from the results of the clearance study conducted with the 13-week subchronic study.

M. Minute Volume

Respiratory physiology data will be collected, using plethysmographs, between 1 hour after the start of exposure and the end of the exposure period for 6 mice/sex/group selected at random from the core study animals.

Tidal volume (ml) and respiration rate (breaths/min) will be monitored continuously for 10 minutes. Average tidal volume and respiration rates will be calculated and stored for all breaths occurring during each 10-second interval of monitoring. Minute volume (ml/min) will be calculated as the product of the 10-second average tidal volume and the 10-second average respiration rate. The mean and standard deviation of the minute volume for the entire 10-minute recording session will then be calculated from the 10-second average minute volumes.

Body weights will be recorded on these mice on the day that the minute volume data are collected. If minute volume data are not collected on a scheduled weighing day, these weights will be in addition to the regular weekly body weights.

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XXII. POSTMORTEM OBSERVATIONS AND MEASUREMENTS

A. Necropsy

A complete necropsy will be performed on all core study mice found dead, moribund sacrificed or at terminal sacrifice. Findings will be recorded on an Individual Animal Necropsy Record (IANR) form. All surviving mice will be necropsied on the day following the last day of exposure. Mice will be killed by anesthetization with CO_2 followed by exsanguination. Necropsies will include an external examination of all body orifices, an examination of tissues/organs, and fixation in 10% neutral buffered formalin (NBF) of the following tissues:

Adrenals
Brain (medulla/pons, cerebellar cortex, cerebral cortex)
Ceacum
Colon
Duodenum
Esophagus
Eyes
Femur (including joint)
Gallbladder
Harderian glands
Heart/aorta
Ileum
Jejunum
Kidneys
Liver
Lungs (trachea, larynx, tongue, pharynx)
Lymph nodes
Mammary gland/skin
Nose
Oral Cavity
Ovaries
Pancreas
Pituitary
Preputial/clitoral glands
Prostate
Rectum
Salivary glands
Sciatic nerve
Seminal vesicles/coagulation glands
Spinal cord (cervical, thoracic, lumbar)
Spleen
Sternum with bone marrow
Stomach
Testes/epididymis
Thigh muscle
Thymus
Thyroid (including parathyroids)
Tissue masses/tumors
Urinary bladder
Uterus
Zymbal's gland

B. Organ Weights

Organ weights will be recorded from all core study mice for both adrenal glands (0.001 g), brain (0.01 g), epididymides (0.001 g), heart (0.01g), kidneys (0.01g.), liver (0.01 g), lung with trachea (0.01 g), spleen (0.01 g), and testes (0.01 g). Organ to body weight and organ to brain weight ratios will be calculated. Organ weights from moribund/humane sacrifices will be collected and reported, but not included in the data analysis.

C. Histopathology

Organs/tissues collected from the filtered air control and the high concentration RASS and DEE groups will be processed to slides and stained with hematoxylin and eosin for histopathologic examination. Special stains will be used at the discretion of the pathologist. Data will be entered onto the Xybion Path/Tox System for all mice.

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A complete histopathologic evaluation inclusive of gross lesions will be performed on all core study mice from the filtered air control and the high concentration RASS and DEE groups sacrificed at the end of the exposure period. Histopathologic evaluation will be conducted on gross lesions and designated tissues from all core study animals. Tissues exhibiting effects in the high concentration group(s) will be examined in the lower concentration group(s) and in the animals sacrificed at the end of the recovery period to a no effect level. Slides may be peer reviewed by the Sponsor. A complete histopathologic evaluation will be conducted on the following tissues:

Accessory genital organs (prostate, seminal vesicles, coagulation glands)
Adrenals, right and left
Aorta (thoracic)
Brain (cerebral cortex, cerebellar cortex, pons/medulla oblongata)
Caecum
Clitoral glands
Colon
Duodenum
Epididymis
Eyes with optic nerve (if grossly abnormal)
Femur, including joint
Harderian glands
Heart
Ileum
Jejunum
Kidney, right and left
Larynx/laryngopharynx (step sections, 4 levels, laryngopharynx at the base of epiglottis)
Liver, gallbladder
Lungs (6-µm sections every 5 mm)
Lymph nodes (mandibular, mesenteric, bronchial and mediastinal)
Mammary glands
Musculature (thigh)
Nose/nasopharynx (4 levels; Young, 1981)
Oral cavity
Ovaries (mesovaries), right and left
Pancreas
Parathyroid glands
Peripheral nerve (sciatic)
Pituitary gland
Preputial glands
Rectum
Salivary glands (submandibular and sublingual)
Skin
Spinal cord (cervical, thoracic, and lumbar)
Spleen
Sternum with bone marrow
Stomach (forestomach and glandular stomach)
Testes, right and left
Thymus
Thyroid glands
Tongue
Trachea
Urinary bladder
Uterus
Zymbal's glands
All grossly visible tumors or lesions suspected being tumors

D. Statistical Analysis

Results from this study will include incidence tables of clinical signs, gross lesions, and histopathological observations. Group means will be calculated for:

Body weight
Body weight gain
Organ weight
Organ:body weight ratio
Organ:brain weight ratio
Leukocyte parameters
Nicotine and cotinine levels
Lung clearance

Any data set with a sample size less than six will have only means and standard deviations calculated. Group variances for body weight, clinical pathology and organ weight data will be compared using Bartlett's test. When the differences between group variances are not significant ($p>0.01$), a one-way analysis of variance (ANOVA) will be performed. If significant differences ($P<0.01$) among the means are indicated by the ANOVA, Dunnett's multiple comparison test will be used to determine the intergroup differences. Dunnett's

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Source: https://www.industrydocuments.ucsf.edu/docs/xslm0000

makes pairwise comparisons among all group means including the control groups. Significance will be declared at the 0.05 and 0.01 alpha levels.

In the event that Bartlett's test indicates significant differences between group variances for a given parameter, the mean values between the control and each concentration group will be compared using the Behrens-Fisher *t*-test (Satterthwaite's method will be used to adjust for degrees of freedom). Significance will be declared at the 0.05 and 0.01 alpha levels if the P value is less than alpha divided by the number of comparisons made (Bonferroni's adjustment for multiple comparisons; Miller 1981).

The percent difference in mean body weights (MBW) between treated and control animals will be calculated as follows:[1]

$$\frac{\text{MBW of Exposed Group - MBW of Control Group}}{\text{MBW of Control Group}} \times 100\%$$

E. Study Conduct and Records Retention

Documentation will be maintained in such a manner that confidentiality for the Sponsor, in all aspects of the study conduct, will be assured.

This protocol will be the controlling document. The Study Director is to be notified immediately for clarification if discrepancies occur between the protocol and the SOPs.

Any changes to this protocol will be made in the following manner. If BNW initiates the change, not associated with cost, verbal (telephonic) approval will be obtained from the Sponsor followed by written documentation by BNW to the Sponsor. Any change which impacts the cost or schedule of the study will be documented in writing and approved by the Sponsor Representative's signature prior to implementation. The Sponsor may initiate modification to the protocol by telephonic authorization to BNW followed by written documentation as stated above.

This study will be conducted in compliance with Good Laboratory Practice regulations, 21 CFR 58. All records required to reconstruct the study will be maintained as stipulated in 21 CFR 58.190. All paraffin blocks and wet tissues resulting from any portion of this study will be retained by BNW until 6 months after acceptance of the final report at which time they will be sent to the Sponsor. If requested by the sponsor, all microscopic slides will be submitted to the Sponsor with the final draft report.

Records to be retained in study archives will be specified by the SOP entitled "Records to be Retained for Philip Morris Studies". Records that accumulate during the study will be retained at BNW until they are shipped to the Sponsor immediately prior to the termination of the contract or until the Sponsor requests transfer, whichever occurs first.

F. Report

Following a Quality Assurance audit of the raw data and data tables, a complete and detailed draft report will be submitted to the Sponsor within the time frame of the contract.

G. References

Miller, R.G. Jr. 1981. *Simultaneous Statistical Inference.* Springer-Verlag, New York, NY.

[1]This procedure is currently in use by the National Toxicology Program, NIEHS, Research Triangle Park, NC.

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Source: https://www.industrydocuments.ucsf.edu/docs/xslm0000

XXIII. BNW APPROVAL

Earl W. Morgan, DVM, ACVPM, DABT
Principal Investigator

Date

R.A. Gelman, MS
Quality Assurance Auditor

Date

XXIV. SPONSOR APPROVAL

George J. Patskan, PhD
Study Monitor

Date

Sponsor's Representative

Date

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THIRTEEN-WEEK NOSE-ONLY INHALATION PILOT STUDY IN MICE

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Source: https://www.industrydocuments.ucsf.edu/docs/xslm0000

THIRTEEN-WEEK NOSE-ONLY INHALATION PILOT STUDY IN MICE

SPONSOR:
Philip Morris
Research Center
P.O. Box 26583
Richmond, Virginia 23261

CONTRACT LABORATORY:
Battelle, Pacific Northwest Laboratories (BNW)
Life Sciences Laboratory (LSL-II Building)
P.O. Box 999
Richland, Washington 99352

Protocol Date: _____

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Source: https://www.industrydocuments.ucsf.edu/docs/xslm0000

I. STUDY TITLE

Thirteen-Week Nose-Only Inhalation Pilot Study in Mice

II. PROJECT OBJECTIVE

The objective of this study is to determine the adaptability of C57BL/6 mice to the stress of a 6 hour per day, 5 days per week, nose-only inhalation exposure for thirteen weeks. The C57BL/6 mouse is the preferred strain for a two-year carcinogenicity bioassay to be conducted on the carcinogenic potential of RASS in mice. The study will include B6C3F1 and CD-1 mice for comparison. Results of this study will be used to select the strain of mice to be used for a two-year carcinogenicity bioassay of RASS and DEE in mice.

III. STUDY SCHEDULE

A. Arrival - week of

B. Start of exposure -

C. Last day of exposure -

IV. PROJECT NUMBER

V. PROTOCOL DATE

VI. SPONSOR

Philip Morris
Research Center
P.O. Box 26583
Richmond, Virginia 23261

VII. REPRESENTATIVE

George J. Patskan, PhD

VIII. TESTING FACILITY

Battelle, Pacific Northwest Laboratories (BNW)
Toxicology Department K4-10
P.O. Box 999
Richland, Washington 99352

IX. STUDY DIRECTOR AND PRINCIPAL INVESTIGATOR

Earl W. Morgan, DVM, ACVPM, DABT

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Source: https://www.industrydocuments.ucsf.edu/docs/xslm0000

X. INHALATION EXPOSURE SYSTEM

A. Exposure Room

Room ____, LSL-II Building

1. Exposure Unit

Cannon flow-past nose-only exposure units. Each unit has 104 exposure ports, 90 of which will be used for rat exposures. The remaining ports will be used for sampling or closed off. The exposure unit is traditionally contained in a rigid, clean plastic cabinet to avoid contamination of the room by the test article aerosol. The sample system will be used in this case, although the test article is filtered air. Airflow through the hood will be maintained at approximately 15 cfm to assure proper cooling of the mice in the restraint tubes. The exposure unit can be rotated so that all mice may be easily observed during exposure.

XI. TEST SYSTEM

A. Specifications

Species:	Mus musculus
Strain:	C57BL/6 (VAF) B6C3F1 (VAF) CD-1 (VAF)
Source:	Charles River Laboratories Raleigh, NC
Age at Arrival:	4-5 weeks
Age at Study Start:	6-7 weeks
Total Number of Animals:	45 males; 45 females
Exposure Tube Acclimation Period:	7-10 days
Identification:	Tail tattoo

B. Reason for Selection

The C57BL/6 (VAF) mouse was selected for a 2-year carcinogenicity bioassay in mice of RASS in mice on the basis of data available in the literature in which this is the only strain of mice in which carcinogenisis has been reported for both RASS and DEE. The B6C3F1 (VAF) and CD-1 (VAF) are being included for comparative purposes and evaluation as possible alternatives in the event that the C57BL/6 dose not adapt to the nose-only restraint tubes for prolonged periods of time.

C. Randomization

The mice which adapt to the exposure tubes will be randomly assigned to exposure groups by using body weight as a blocking variable to ensure that there are no statistically significant differences in initial group mean body weights. The weight distribution range (by sex) of the mice selected for the study will be as narrow as possible. A sufficient quantity of mice will be ordered and adapted to the exposure restraint tubes to ensure that no rat will be selected whose body weight exceeds ±20% of the mean (by sex) at the start of the study. The XYBION PATH/TOX System (XYBION Medical Systems Corporation, Cedar Knolls, NJ) will be used for randomization.

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Source: https://www.industrydocuments.ucsf.edu/docs/xslm0000

D. Housing and Maintenance

Mice shipping crates will be examined upon arrival for evidence of conditions likely to permit exposure to pathogens (soiled, wet, or otherwise damaged). The uncrating will be conducted at the door of the animal room. While being removed from the crates, the mice will be examined for evidence of shipping stress.

The mice will be acclimated in room —— of the LSL-II Building for 10-14 days prior to the start of exposure. Mice will remain on quarantine status until health screen procedures are completed, approximately 3 weeks after their arrival.

Throughout the study, cage space will meet the requirements stated in the 85-23 (1985) NIH "Guide for Care and Use of Laboratory Animals". The BNW animal care and use program, including the facility used for this study, is accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

The mice will be housed approximately 5 mice per cage in hanging wire cages prior to identification and randomization at which time they will be housed individually in stainless steel wire cage units on stainless steel racks. Mice will be individually housed for the remainder of the study.

Room temperatures during the acclimatization and study will be maintained at 75 ± 2°F and relative humidities at 55 ± 15%. Measurements will be recorded at ~1 hour intervals.

Twelve hours light and twelve hours dark will be maintained with light starting at 0600.

The clinical veterinarian will make a visual inspection of the mice to be used in the study just prior to their release for the study. Five mice per sex that were not used for the exposures will be evaluated for evidence of disease 18-21 days after arrival at BNW. Gross necropsy, histopathology, and serological testing for evidence of disease and the presence of potentially pathogenic organisms will be performed. Sera will be tested for antibodies to Sendai virus, mouse hepatitis virus, GDVII virus, minute virus of mice, pneumonia virus of mice (PVM) and Mycoplasma pulmonis. Mice will be released from quarantine if the health screen doesn't provide evidence of pathogens or disease.

Serological evaluations will be performed on 5 mice/sex at the time of terminal sacrifice.

During the acclimation period, approximately 7-10 days prior to the start of exposure, mice will be placed in nose-only exposure restraint tubes for increasing time up to 6 hours daily for five days to allow them to adapt to confinement in the tubes. The mice which adapt to the restraint tubes will then be weighed, randomized, assigned to exposure concentration groups and identified individually with a unique number tail tattoo approximately two days before the exposure period.

Mice not used for the study will be euthanized one week after the start of the study. The disp osition of these mice will be recorded on the Animal Disposition Record and retained in the study files.

Mice which die during the first week of the study will be replaced from the pool of unused mice.

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E. Diet and Water

NIH-07 Open Formula diet will be provided *ad libitum* except during the inhalation exposures when mice are in restraint tubes.

This diet is analyzed for contaminants and some nutrient components; these analyses are reviewed for acceptability using established standards prior to use of the food. There are no known contaminants in the diet which could be expected to alter the outcome of the study.

Fresh softened water (ion exchange softener, Illinois Water Treatment Company, Model 2R-2240, Rockford, IL) will be supplied ad libitum except during exposures. An automatic watering system (Edstrom Industries, Waterford, WI) will be used during the quarantine/acclimatization period and throughout the study. Water bottles will also be provided for the first three days after arrival.

Representative samples of animal drinking water from the LSL-II facility will be analyzed for contaminants at least once per year. There are no known contaminants in the drinking water which could be expected to alter the outcome of the study. The hardness of the water will be checked approximately once every week. Records will be retained in the LSL-II Building Engineer's office.

F. Environmental Monitoring

1. Air Supply

Room and exposure supply air will be HEPA-filtered. Both room and exposure exhaust air will pass through a series of two HEPA filters.

2. Temperatures

Temperatures in each exposure unit cabinet and in the exposure air stream at a nose port will be monitored at ~2-minute intervals and recorded at ~60 minute intervals during the daily exposure period. If the temperature is not within the acceptable range 75 ± 4°F, the system will alarm. Daily means for the exposure cabinet and the exposure atmosphere will be maintained within 75 ± 4°F.

Temperatures in the animal room will be monitored at ~2-minute intervals and recorded once every hour. If the temperature is not within the acceptable range 75 ± 2°F, the system will alarm. The acceptable range for individual animal room measurements and the daily mean will be 75 ± 2°F.

3. Relative Humidity

Relative humidity will be monitored at a nose-port in each exposure unit and recorded at ~60 minute intervals during the exposure period. If the relative humidity is not within the acceptable range 55 ± 15%, the system will alarm. Daily means for the exposure unit will be maintained within 55 ± 15%.

Animal room humidity will be monitored and recorded once every hour. If the relative humidity is not within the acceptable range 55 ± 15%, the system will alarm. The acceptable daily mean is 55 ± 15% relative humidity.

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4. Airflow

Airflow will be monitored at an inlet orifice using a calibrated photohelic pressure gauge. Flow will be recorded once per hour during the exposure period. The acceptable minimum flow is 250 ml/min per occupied exposure port.

Measurements out of specified limits will be recorded and result in an alarm so that the operator can take appropriate actions.

XII. EXPERIMENTAL DESIGN

A. Route of Administration and Reason for Selection

For the planned two-year carcinogenicity bioassay the test articles will be administered by nose-only inhalation. The concentration of RASS and the route of administration were selected on the basis of route of human exposure. This study is designed to evaluate the ability of the selected strains of mice to adapt to the stress of confinement in the nose-only exposure tubes for 6 hours per day, 5 days per week (excluding holidays) for 13 weeks.

B. Experimental Design

Treatment	Number of Strains	Number of Mice/Strain	Daily Observations[a]	Clinical Signs[a]	Body Weights[b]	Total
Filtered Air	3	15M	15M	15M	15M	45M
	3	15F	15F	15F	15F	45F

[a]All survivors to that study day (twice daily for moribundity, mortality, and clinical signs)
[b]All survivors to scheduled weighing day

C. Animal Identification

Strain	Study Mice[a]
C57BL/6	1-15 (M) 101-115 (F)
B6C3F1	201-215 (M) 301-315 (F)
CD-1	401-415 (M) 501-515 (F)

1. Route of Administration

Nose-only inhalation

2. Frequency/Duration

Six hour/day; 5 days/week; at least 13 weeks, excluding holidays and weekends

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3. Daily Observations

Twice daily for moribundity and mortality on all surviving mice.

4. Body Weights

Weekly on all surviving mice.

5. Clinical Signs

Weekly for all survivors and prior to unscheduled sacrifice for moribund or humane sacrifice mice.

D. Postmortem Observations and Measurements

1. Necropsy

A complete necropsy will be performed only on mice sacrificed as moribund or at termination of the study on mice exhibiting gross signs of stress. Findings will be recorded on an Individual Animal Necropsy Record (IANR) form. Mice, as needed, will be necropsied on the day following the last day of exposure. Mice will be killed by anesthetization with CO_2 followed by exsanguination. Necropsies will include an external examination of all body orifices, an examination of tissues/organs, and fixation in 10% neutral buffered formalin (NBF) only on tissues exhibiting gross abnormalities potentially related to stress.

2. Organ Weights

Testicular weight (0.01 g) will be recorded on all males. Additional organ weights will only be recorded from organs exhibiting gross signs.

3. Histopathology

Will only be performed at the discretion of the pathologist to evaluate the potential effects of stress. Data will be entered onto the Xybion Path/Tox System.

E. Statistical Analysis

Results from this study will include incidence tables of clinical signs, gross lesions and histopathological observations, if performed.

Group means will be calculated for:

Body weight
Body weight gain
Testicular weight

Any group with a sample size less than six will have only means and standard deviations calculated. Group variances for body weight and testicular weight data will be compared using Bartlett's test. When the differences between group variances are not significant ($p>0.01$), a one-way analysis of variance (ANOVA) will be performed. If significant differences ($P<0.01$) among the means are indicated by the ANOVA, Dunnett's multiple comparison test will be used to determine the intergroup differences. Dunnett's makes pairwise comparisons among all group means including the control groups. Significance will be declared at the 0.05 and 0.01 alpha levels.

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Source: https://www.industrydocuments.ucsf.edu/docs/xslm0000

In the event that Bartlett's test indicates significant differences between group variances for a given parameter, the mean values between the control and each concentration group will be compared using the Behrens-Fisher *t*-test (Satterthwaite's method will be used to adjust for degrees of freedom). Significance will be declared at the 0.05 and 0.01 alpha levels if the P value is less than alpha divided by the number of comparisons made (Bonferroni's adjustment for multiple comparisons; Miller 1981).

The percent difference in mean body weights (MBW) between treated and control animals will be calculated as follows:[1]

$$\frac{\text{MBW of Exposed Group - MBW of Control Group}}{\text{MBW of Control Group}} \times 100\%$$

F. Study Conduct and Records Retention

Documentation will be maintained in such a manner that confidentiality for the Sponsor, in all aspects of the study conduct, will be assured.

This protocol will be the controlling document. The Study Director is to be notified immediately for clarification if discrepancies occur between the protocol and the SOPs. Any changes to this protocol will be made in the following manner. If BNW initiates the change, not associated with cost, verbal (telephonic) approval will be obtained from the Sponsor followed by written documentation by BNW to the Sponsor. Any change which impacts the cost or schedule of the study will be documented in writing and approved by the Sponsor Representative's signature prior to implementation. The Sponsor may initiate modification to the protocol by telephonic authorization to BNW followed by written documentation as stated above.

This study will be conducted in compliance with Good Laboratory Practice regulations, 21 CFR 58. All records required to reconstruct the study will be maintained as stipulated in 21 CFR 58.190. All paraffin blocks and wet tissues resulting from any portion of this study will be retained by BNW until 6 months after acceptance of the final report at which time they will be sent to the Sponsor. If requested by the sponsor, all microscopic slides will be submitted to the Sponsor with the final draft report.

Records to be retained in study archives will be specified by the SOP entitled "Records to be Retained for Philip Morris Studies". Records that accumulate during the study will be retained at BNW until they are shipped to the Sponsor immediately prior to the termination of the contract or until the Sponsor requests transfer, whichever occurs first.

G. Report

Following a Quality Assurance audit of the raw data and data tables, the results of this study will be included in the phase one report.

H. References

Miller, R.G. Jr. 1981. Simultaneous Statistical Inference. Springer-Verlag, New York, NY.

[1]This procedure is currently in use by the National Toxicology Program, NIEHS, Research Triangle Park, NC.

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Source: https://www.industrydocuments.ucsf.edu/docs/xslm0000

XIII. BNW APPROVAL

Earl W. Morgan, DVM, ACVPM, DABT
Principal Investigator

Date

R.A. Gelman, MS
Quality Assurance Auditor

Date

XIV. SPONSOR APPROVAL

George J. Patskan, PhD
Sponsor's Representative

Date

2023685878

Source: https://www.industrydocuments.ucsf.edu/docs/xslm0000

THIRTEEN-WEEK NOSE-ONLY INHALATION TOXICITY STUDY OF ROOM AGED CIGARETTE SIDESTREAM SMOKE AND DIESEL ENGINE EXHAUST IN MICE

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Source: https://www.industrydocuments.ucsf.edu/docs/xslm0000

THIRTEEN-WEEK NOSE-ONLY INHALATION TOXICITY STUDY OF ROOM AGED CIGARETTE SIDESTREAM SMOKE AND DIESEL ENGINE EXHAUST IN MICE

SPONSOR:
Philip Morris
Research Center
P.O. Box 26583
Richmond, Virginia 23261

CONTRACT LABORATORY:
Battelle, Pacific Northwest Laboratories (BNW)
Life Sciences Laboratory (LSL-II Building)
P.O. Box 999
Richland, Washington 99352

Protocol Date: _____

2023685880

Source: https://www.industrydocuments.ucsf.edu/docs/xslm0000

I. STUDY TITLE

Thirteen-Week Nose-Only Inhalation Toxicity Study of Room Aged Cigarette Sidestream Smoke and Diesel Engine Exhaust in Mice

II. PROJECT OBJECTIVE

Objectives of this study are to determine the toxicity of RASS and DEE administered by nose-only inhalation to male and female mice 6 hours per day, 5 days per week, for thirteen weeks to evaluate the system, to work out technical difficulties, and to establish laboratory procedures. The study will include groups of mice to be used for studies of potential carcinogenicity mechanisms. The study will also provide data on an additional group which will be allowed to recover for 13 weeks. Results of this study will be used to validate the doses of RASS for a 2-year carcinogenicity bioassay in mice and to select the dose of DEE to be used as the positive control in that study.

III. STUDY SCHEDULE

A. Arrival - week of

B. Start of exposure -

C. Last day of exposure -

IV. PROJECT NUMBER

V. PROTOCOL DATE

VI. SPONSOR

Philip Morris
Research Center
P.O. Box 26583
Richmond, Virginia 23261

VII. SPONSOR'S REPRESENTATIVE

George J. Patskan, PhD

VIII. TESTING FACILITY

Battelle, Pacific Northwest Laboratories (BNW)
Toxicology Department K4-10
P.O. Box 999
Richland, Washington 99352

IX. STUDY DIRECTOR AND PRINCIPAL INVESTIGATOR

Earl W. Morgan, DVM, ACVPM, DABT

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Source: https://www.industrydocuments.ucsf.edu/docs/xslm0000

X. TEST ARTICLE DESCRIPTION

A. Identification

1. Room Aged Sidestream Smoke (RASS)

RASS will be obtained by aging sidestream smoke (SS) for 0.5 hours (mean age) in a room. SS will be generated from the standard reference filter cigarette, 1R4F, smoked in basic conformity with ISO standards 3402, 4387, and 3308 (1991). The cigarettes will be smoked by a 30-port smoking machine (CH Technologies, Westwood, NJ).

B. Lot Number

C. How Supplied

D. Storage Conditions

Cigarettes will be stored in a freezer at -16 ± 2°C. They will be preconditioned for at least 48 hours at room conditions (22 ± 2°C and 60 ± 10% RH) prior to smoking. Distribution and handling during the course of the study will be conducted in such a manner that proper identification will be maintained and contamination avoided.

E. Source

Philip Morris

XI. POSITIVE CONTROL DESCRIPTION

A. Diesel Engine Exhaust

The DEE will be generated from a light-duty pickup truck engine, mounted on a shock mount engine stand attached to a dynamometer system. The dynamometer will be programmed to simulate the EPA Urban Dynamometer Driving Schedule (40CFR Ch. 1, Pt. 86, App I).

B. Storage Condition

Diesel fuel will be stored in tank external to the building with a small day tank in the generator room.

C. Source

XII. TEST ATMOSPHERE

On-line monitoring of the mass concentration of RASS and DEE will be accomplished by two Tapered Element Oscillating Microbalance Mass Monitors (TEOM 1400, Rupprect & Patashnick Co., Inc., Albany, NY). The TOEMs will sample RASS and DEE aerosols from a representative exposure port in the exposure unit. Exposure concentrations will be controlled to within ±20 % of target concentration by adjustment of the exposure unit dilution airflow.

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Source: https://www.industrydocuments.ucsf.edu/docs/xslm0000

A real time aerosol monitor (RAM; MIE, Inc., Bedford, MA) will be used to monitor the concentration of RASS as it enters the distribution line from the aging room. Aerosol concentrations will be monitored with the RAM twice per hour over the duration of the daily generation period. The relative response of the RAM to the aerosol concentration (volts per unit concentration) will be determined prior to the start of exposure. RAM response will be evaluated by comparing the measured RAM voltage to the aerosol concentration of RASS determined from independent filter samples.

The total particulate mass (TPM) of both RASS and DEE in the test atmosphere during the exposure will be determined from duplicate Cambridge filter samples from both the distribution line at the exit from the aging room and two exposure ports from each exposure unit collected over the duration of the generation period. These filters will be analyzed gravimetrically and/or by UV analysis. Filter sample extracts will be analyzed as soon as feasible following collection and preparation.

The buildup and decay of the test article concentration in the exposure unit will be determined prior to start of the study. The time to buildup to 90% of the target concentration (T_{90}) and the time to decay to 10% of the target concentration (T_{10}) will be determined.

The particle size distribution of the aerosol in the exposure unit will be measured prior to the start of exposure and once a month during the exposure phase. Median diameters and geometric standard deviations (GSD) will be reported.

Uniformity of the concentration and particle size distribution of the test article in the nose-only exposure unit will be determined during the prestart phase of the study. Uniformity of test article concentration will be demonstrated by monitoring one port on each level with the TEOM or RAM. Uniformity of particle size distribution will be demonstrated by samples taken at the top, middle and bottom levels of the exposure unit.

XIII. INHALATION EXPOSURE SYSTEM

A. Exposure Room

Rooms 523 and 531, LSL-II Building

B. Exposure Unit

Cannon flow-past nose-only exposure units. Each unit has 104 exposure ports, 90 of which will be used for rat exposures. The remaining ports will be used for sampling or closed off. Each exposure unit will be contained in a rigid clear plastic cabinet to assure no contamination of the room by the test article aerosol. Airflow through the hood will be maintained at approximately 15 cfm to assure proper cooling of the mice in the restraint tubes. The exposure unit can be rotated so that all mice may be easily observed during exposure.

C. Exposure Generator

RASS will be obtained by aging sidestream smoke (SS) for 0.5 hours (mean age) in a 30 m^3 room. SS will be generated from the standard reference filter cigar ette, 1R4F, smoked by a 30-port smoking machine (CH Technologies; Westwood, NJ) in basic conformity with ISO standards 3402, 4387, and 3308 (1991). Mainstream smoke from the smoking machine will be routed through a filter system to remove the majority of the particulate material prior to being routed to the building exhaust. A stainless steel cone will be fitted over the smoking machine to extract the SS which will be mixed with conditioned dilution air (22 ± 2°C and 60 ± 10% RH) before being injected into a smoke aging room by a flow-controlled fan. The

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Source: https://www.industrydocuments.ucsf.edu/docs/xslm0000

entire flow from the aging room will be delivered to the exposure rooms via a stainless steel delivery tube. The required flow of RASS will be siphoned from this delivery line by a pressurized-air-controlled ejector tube, mixed with conditioned air to achieve the proper concentration, and injected into the nose only exposure unit. Exhaust from the exposure units will be routed through a filter system to remove the majority of the particulate material prior to being routed to the building exhaust.

DEE will be generated from a light-duty pickup truck engine, mounted on a shock mount engine stand attached to a dynamometer system. The dynamometer will be programmed to simulate the EPA Urban Dynamometer Driving Schedule (40CFR Ch. 1, Pt. 86, App I). A constant flow of DEE from the engine will be delivered from a plenum downstream of the engine muffler to the exposure rooms through a heated stainless-steel deliver line. The required flow of DEE will be siphoned from this delivery line by a pressurized-air-controlled ejector tube, mixed with conditioned air to achieve the proper concentration, and injected into the nose only exposure unit. All DEE exhaust will be routed to the building exhaust.

XIV. TEST SYSTEM SPECIFICATIONS

Species:	Mus musculus
Strain:	TBD (VAF)
Source:	Charles River Laboratories Raleigh, NC
Age at Arrive:	4-5 weeks
Age at Study Start:	6-7 weeks
Total Number of Animals:	275 males; 185 females (includes 55M/55F Big Blue mice)
Exposure Tube Acclimation Period:	~5 days
Identification:	Tail tattoo

XV. REASON FOR SELECTION

The ____ mouse was selected for this study on the basis of data available in the literature and a 13-week pilot study.

XVI. RANDOMIZATION

The mice which adapt to the exposure tubes will be randomly assigned to exposure groups by using body weight as a blocking variable to ensure that there are no statistically significant differences in initial group mean body weights. The weight distribution range (by sex) of the mice selected for the study will be as narrow as possible. A sufficient quantity of mice will be ordered and adapted to the exposure restraint tubes to ensure that no mouse will be selected whose body weight exceeds ±20% of the mean (by sex) at the start of the study. The Xybion Path/Tox System (Xybion Medical Systems Corporation, Cedar Knolls, NJ) will be used for randomization.

XVIII. HOUSING AND MAINTENANCE

Mice shipping crates will be examined upon arrival for evidence of conditions likely to permit exposure to pathogens (soiled, wet, or otherwise damaged). The uncrating will be conducted at the door of the animal room. While being removed from the crates, the mice will be examined for evidence of shipping stress.

The mice will be acclimated in Room ____ of the LSL-II Building for 2 weeks prior to the start of exposure. Mice will remain on quarantine status until health screen procedures are completed, approximately 3 weeks after their arrival.

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Throughout the study, cage space will meet the requirements stated in the 85-23 (1985) NIH "Guide for Care and Use of Laboratory Animals". The BNW animal care and use program, including the facility used for this study, is accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

The mice will be housed approximately 5 mice per cage in hanging wire cages prior to identification and randomization at which time they will be housed individually in stainless steel wire cage units on stainless steel racks. Mice will be individually housed for the remainder of the study.

Room temperatures during the acclimatization and study will be maintained at 75 ± 2°F and relative humidities at 55 ± 15%. Measurements will be recorded at ~1 hour intervals.

Twelve hours light and twelve hours dark will be maintained with light starting at 0600.

The clinical veterinarian will make a visual inspection of the mice to be used in the study just prior to their release for the study. Mice will be released for study if the health screen doesn't provide evidence of pathogens or disease. Five mice per sex that were not used for the exposures will be reevaluated for evidence of disease 18-21 days after arrival at BNW. Sera will be tested for antibodies to Sendai virus, mouse hepatitis virus, GDVII virus, minute virus of mice, pneumonia virus of mice (PVM) and *Mycoplasma pulmonis*.

Sentinel mice (5M,5F) will be selected from the extra mice prior to the start of exposures. Serological evaluations will be performed on these mice at the time of terminal sacrifice of the core study mice.

During the acclimation period, approximately 7-10 days prior to the start of exposure, mice will be placed in nose-only exposure restraint tubes for increasing time up to 6 hours daily to allow them to adapt to confinement in the tubes. The mice which adapt to the restraint tubes will then be weighed, randomized, assigned to exposure concentration groups and identified individually with a unique number tail tattoo approximately two days before the exposure period.

Mice not used for the study will be euthanized one week after the start of the study. The disposition of these mice will be recorded on the Animal Disposition Record and retained in the study files.

Mice which die during the first week of the study will be replaced from the pool of unused mice.

XVIII. DIET AND WATER

NIH-07 Open Formula diet will be provided *ad libitum* except during the inhalation exposures when mice are in restraint tubes.

This diet is analyzed for contaminants and some nutrient components; these analyses are reviewed for acceptability using established standards prior to use of the food. There are no known contaminants in the diet which could be expected to alter the outcome of the study.

Fresh softened water (ion exchange softener, Illinois Water Treatment Company, Model 2R-2240, Rockford, IL) will be supplied *ad libitum* except during exposures. An automatic watering system (Edstrom Industries, Waterford, WI) will be used during the quarantine/acclimatization period and throughout the study. Water bottles will also be provided for the first three days after arrival.

Representative samples of animal drinking water from the LSL-II facility will be analyzed for contaminants at least once per year. There are no known contaminants in the drinking water which

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could be expected to alter the outcome of the study. The hardness of the water will be checked approximately once every week. Records will be retained in the LSL-II Building Engineer's office.

XIX. ENVIRONMENTAL MONITORING

A. Air Supply

Room and exposure supply air will be HEPA-filtered. Both room and exposure exhaust air will pass through a series of two HEPA filters.

B. Temperatures

Temperatures in each exposure unit cabinet and in the exposure air stream at a nose port will be monitored at ~2-minute intervals and recorded at ~60 minute intervals during the daily exposure period. If the temperature is not within the acceptable range 75 ± 4°F, the system will alarm. Daily means for the exposure cabinet and the exposure atmosphere will be maintained within 75 ± 4°F.

Temperatures in the animal room will be monitored at ~2-minute intervals and recorded once every hour. If the temperature is not within the acceptable range 75 ± 4°F, the system will alarm. The acceptable range for individual animal room measurements and the daily mean will be 75 ± 4°F.

C. Relative Humidity

Relative humidity will be monitored at a nose-port in each exposure unit and recorded at ~60 minute intervals during the exposure period. If the relative humidity is not within the acceptable range 55±15%, the system will alarm. Daily means for the exposure unit will be maintained within 55±15%.

Animal room humidity will be monitored and recorded once every hour. If the relative humidity is not within the acceptable range 55±15%, the system will alarm. The acceptable daily mean is 55±15% relative humidity.

D. Airflow

Airflow will be monitored at an inlet and exhaust orifices using a calibrated photohelic pressure gauge. Both flows will be recorded once per hour during the exposure period. The acceptable minimum inlet flow is 250 ml/min per occupied exposure port.

Measurements out of specified limits will be recorded and result in an alarm so that the operator can take appropriate actions.

XX. EFFLUENT TREATMENT

Exposure unit exhaust will be passed through a series of two HEPA filters and a catalytic converter to remove all test article. The concentration of test article in the building exhaust will be measured once during the study to determine the efficiency of the effluent treatment system.

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XXI. EXPERIMENTAL DESIGN

A. Route of Administration and Reason for Selection

The test articles will be administered by nose-only inhalation at concentrations of 1 and 10 mg/m^3 of RASS and 3 and 10 mg/m^3 of DEE. A filtered air control group will be included. The concentration of RASS and the route of administration used in this study were selected on the basis of route of human exposure and to provide target particle concentrations which exceed those determined for extreme human exposure to environmental tobacco smoke by a factor of approximately 10. The DEE concentrations were selected on the basis of data in the literature which indicate that these concentrations should result in a measurable carcinogenic response in the mouse.

B. Experimental Design

Group Number	Exposure Concentration (mg/m^3)	Core Study Mice[a,b,c]	Blue Mouse Micronucleus Assay Mice	Enzyme Assay Mice	Adducts Assay Mice	Plumonary Clearance Mice	Total
1	0	15M 15F	10M 10F	6M 6F	5M 5F	18M	54M 36F
2	1 (RASS)	15M 15F	10M 10F	6M 6F	5M 5F	18M	54M 36F
3	10 (RASS)	15M 15F	10M 10F	6M 6F	5M 5F	18M	54M 36F
4	3 (DEE)	15M 15F	10M 10F	6M 6F	5M 5F	18M	54M 36F
5	10 (DEE)	15M 15F	10M 10F	6M 6F	5M 5F	18M	54M 36F
Sentinels	0	5M 5F	– –	– –	5M 5F	– –	5M 5F

[a]Includes 5 mice which will be used for a 13 week recovery period.
[b]Blood will be collected from 5/sex/group for carboxyhemoglobin determination.
[c]Urine will be collected from 5/sex from controls and RASS treated mice for nicotine and cotinine determination.

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C. Animal Identification

Group Number	Exposure Concentration (mg/m^3)	Core Study Mice	Blue Mouse Micronucleus Assay Mice	Enzyme Assay Mice	Adducts Assay Mice	Plumonary Clearance Mice
1	0	1-15 (M) 101-115 (F)	16-25 (M) 116-125 (F)	26-31 (M) 26-31 (F)	32-36 (M) 32-36 (F)	37-54 (M) 137-154 (F)
2	1 (RASS)	201-215 (M) 301-315 (F)	216-225 (M) 316-325 (F)	226-231 (M) 326-331 (F)	232-236 (M) 332-336 (F)	237-254 (M) 337-354 (F)
3	10 (RASS)	401-415 (M) 501-515 (F)	416-425 (M) 516-525 (F)	426-431 (M) 526-531 (F)	432-436 (M) 532-536 (F)	437-454 (M) 537-554 (F)
4	3 (DEE)	601-615 (M) 701-715 (F)	616-625 (M) 716-725 (F)	626-631 (M) 726-731 (F)	632-636 (M) 732-736 (F)	637-654 (M) 737-754 (F)
5	10 (DEE)	801-815 (M) 901-915 (F)	816-825 (M) 916-925 (F)	826-831 (M) 926-931 (F)	832-836 (M) 932-936 (F)	837-854 (M) 937-954 (F)
Sentinels	0	SM1-SM5 SF1-SF5	– –	– –	– –	– –

D. Route of Administration

Nose-only inhalation

E. Frequency/Duration

Six hours/day plus T_{90}; 5 days/week; at least 13 weeks, excluding holidays and weekends

F. Daily Observations

Twice daily for moribundity and mortality on all surviving mice.

G. Clinical Signs

Weekly for all survivors and prior to unscheduled sacrifice for moribund or humane sacrifice mice.

H. Body Weights

Weekly on all surviving mice.

I. Urine Nicotine/Continine

During week 4 for 5/sex/RASS treated and filtered air control groups selected at random from the core study animals. Mice will be placed in metabolism cages for a 16 hour urine collection.

J. Micronucleus and Big Blue™ Mouse Assay

During week 5, the 10 Big Blue mice/sex/group will be anesthetized and the lungs perfused and removed aseptically. One-half of each lung will be used for the lung fibroblast micronucleus assay and the other half will be used for the Big Blue Mouse in vivo

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mutagenic assay. Slides or plates from 5 each from the 10 mg/m^3 RASS and DEE treated groups and the filtered air control group will be analyzed. If a high dose treated group(s) exhibits a positive response, then the slides and/or plates for the low dose group(s) will be scored.

K. Enzyme Activity

During week 4, lung, liver, and kidney samples will be collected from 6 mice/sex/group. Microsomal and cytosolic subcellular fractions will be prepared by differential centrifugation. Cytochrome P450 and glutathione-S-transferase activity will be determined.

L. Protein/DNA Adducts

Blood will be collected from 5 mice/sex/group from the adducts assay special study animals prior to terminal sacrifice. The mice will be placed in metabolism cages for a 16 hour urine collection. At terminal sacrifice samples of the lung and trachea will be collected from these animals and frozen at -80°C. The urine and tissue samples will be evaluated for protein/DNA adducts.

M. Carboxyhemoglobin

Blood will be collected from 3/sex/group selected at random from the core study animals during weeks 5, 9, and 13. Mice will be removed from the exposure chamber within one hour before the end of the daily exposure period, anesthetized with ~70% CO_2, and bled from the supra-orbital sinus.

K. Clinical Pathology Evaluations

Prior to terminal necropsy, 10 mice/sex/group selected at random from the core study animals will be anesthetized with ~70% CO_2 and blood samples will be collected from the supra-orbital area into tubes containing potassium EDTA for a leukocyte differential count.

L. Lung Clearance

On the day following the termination of the 13-week RASS and DEE exposures, 18 male mice/group will be exposed to 10 mg TiO_2/m^3 for 5 consecutive days. Lung burdens of TiO_2 will be determined, using ICP-AES techniques, on 3 animals/group at 0, 1, 3, 9, 30, and 90 days following termination of TiO_2 exposures.

M. Minute Volume

Six male mice per group (one group/day) during the TiO_2 exposures. Collection of respiratory data from the plethysmographs will be conducted between 1 hour after the start of exposure and the end of the exposure period.

Tidal volume (ml) and respiration rate (breaths/min) will be monitored continuously for 10 minutes. Average tidal volume and respiration rates will be calculated and stored for all breaths occurring during each 10-second interval of monitoring. Minute volume (ml/min) will be calculated as the product of the 10-second average tidal volume and the 10-second average respiration rate. The mean and standard deviation of the minute volume for the entire 10-minute recording session will then be calculated from the 10-second average minute volumes.

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Body weights will be recorded on these mice on the day that the minute volume data are collected. If minute volume data are not collected on a scheduled weighing day, these weights will be in addition to the regular weekly body weights.

N. Proliferating Cell Nuclear Antigen

At histopathology, additional sections of nose, larynx, and lungs will be cut. Mucosal epithelial cell replication rates in these tissues will be compared by labeling the nuclei with the PC-10 monoclonal antibody and counting labeled nuclei.

XXII. POSTMORTEM OBSERVATIONS AND MEASUREMENTS

A. Necropsy

A complete necropsy will be performed on all core study mice. Findings will be recorded on an Individual Animal Necropsy Record (IANR) form. All 13-week study mice will be necropsied on the day following the last day of exposure. The recovery group will be necropsied 13 weeks after the last exposure. Mice will be killed by anesthetization with CO_2 followed by exsanguination. Necropsies will include an external examination of all body orifices, an examination of tissues/organs, and fixation in 10% neutral buffered formalin (NBF) of the following tissues:

Adrenals
Brain (medulla/pons, cerebellar cortex, cerebral cortex)
Cecum
Colon
Duodenum
Esophagus
Eyes
Femur (including joint)
Gallbladder
Harderian glands
Heart/aorta
Ileum
Jejunum
Kidneys
Liver
Lungs (trachea, larynx, tongue, pharynx)
Lymph nodes
Mammary gland/skin
Nose
Ovaries
Pancreas
Pituitary
Preputial/clitoral glands
Prostate
Rectum
Salivary glands
Sciatic nerve
Seminal vesicles/coagulation glands
Spinal cord (cervical, thoracic, lumbar)
Spleen
Sternum with bone marrow
Stomach
Testes/epididymis
Thigh muscle
Thymus
Thyroid (including parathyroids)
Tissue masses/tumors
Urinary bladder
Uterus
Zymbal's gland

B. Organ Weights

Organ weights will be recorded from all core study mice for both adrenal glands (0.001 g), brain (0.01 g), epididymides (0.001 g), heart (0.01g), kidneys (0.01g), liver (0.01 g), lung with trachea (0.01 g), spleen (0.01 g), and testes (0.01 g). Organ to body weight and organ to brain weight ratios will be calculated. Organ weights from moribund/humane sacrifices will be collected and reported, but not included in the data analysis.

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C. Histopathology

Organs/tissues collected from the filtered air control and the high concentration RASS and DEE groups will be processed to slides and stained with hematoxylin and eosin for histopathologic examination. Special stains will be used at the discretion of the pathologist. Data will be entered onto the Xybion Path/Tox System for all mice.

A complete histopathologic evaluation inclusive of gross lesions will be performed on all core study mice from the filtered air control and the high concentration RASS and DEE groups sacrificed at the end of the 13 week exposure period. Histopathologic evaluation will be conducted on gross lesions and designated tissues from all core study animals. Tissues exhibiting effects in the high concentration group(s) will be examined in the low concentration group(s) and in the animals sacrificed at the end of the recovery period. Slides may be peer reviewed by the Sponsor. A complete histopathologic evaluation will be conducted on the following tissues:

Accessory genital organs (prostate, seminal vesicles, coagulation glands)
Adrenals, right and left
Aorta (thoracic)
Brain (cerebral cortex, cerebellar cortex, pons/medulla oblongata)
Caecum
Clitoral glands
Colon
Duodenum
Epididymis
Eyes with optic nerve (if grossly abnormal)
Femur, including joint
Harderian glands
Heart
Ileum
Jejunum
Kidney, right and left
Larynx/laryngopharynx (step sections, 4 levels, laryngopharynx at the base of epiglottis)
Liver, gallbladder
Lungs (6-µm sections every 5 mm)
Lymph nodes (mandibular, mesenteric, bronchial and mediastinal)
Mammary glands
Musculature (thigh)
Nose/nasopharynx (4 levels; Young, 1981)
Oral cavity
Ovaries (mesovaries), right and left
Pancreas
Parathyroid glands
Peripheral nerve (sciatic)
Pituitary gland
Preputial glands
Rectum
Salivary glands (submandibular and sublingual)
Skin
Spinal cord (cervical, thoracic, and lumbar)
Spleen
Sternum with bone marrow
Stomach (forestomach and glandular stomach)
Testes, right and left
Thymus
Thyroid glands
Tongue
Trachea
Urinary bladder
Uterus
Zymbal's glands
All grossly visible tumors or lesions suspected being tumors

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D. Statistical Analysis

Results from this study will include incidence tables of clinical signs, gross lesions, and histopathological observations. Group means will be calculated for:

Body weight
Body weight gain
Organ weight
Organ:body weight ratio
Organ:brain weight ratio
Leukocyte parameters
Nicotine and cotinine levels
Lung clearance
Incidence of micronuclei
Incidence of mutant colonies
Enzyme activity

Any group with a sample size less than six will have only means and standard deviations calculated. Group variances for body weight, clinical pathology and organ weight data will be compared using Bartlett's test. When the differences between group variances are not significant ($p>0.01$); a one-way analysis of variance (ANOVA) will be performed. If significant differences ($P<0.01$) among the means are indicated by the ANOVA, Dunnett's multiple comparison test will be used to determine the intergroup differences. Dunnett's makes pairwise comparisons among all group means including the control groups. Significance will be declared at the 0.05 and 0.01 alpha levels.

In the event that Bartlett's test indicates significant differences between group variances for a given parameter, the mean values between the control and each concentration group will be compared using the Behrens-Fisher t-test (Satterthwaite's method will be used to adjust for degrees of freedom). Significance will be declared at the 0.05 and 0.01 alpha levels if the P value is less than alpha divided by the number of comparisons made (Bonferroni's adjustment for multiple comparisons; Miller 1981).

The percent difference in mean body weights (MBW) between treated and control animals will be calculated as follows:[1]

$$\frac{\text{MBW of Exposed Group - MBW of Control Group}}{\text{MBW of Control Group}} \times 100\%$$

E. Study Conduct and Records Retention

Documentation will be maintained in such a manner that confidentiality for the Sponsor, in all aspects of the study conduct, will be assured.

This protocol will be the controlling document. The Study Director is to be notified immediately for clarification if discrepancies occur between the protocol and the SOPs. Any changes to this protocol will be made in the following manner. If BNW initiates the change, not associated with cost, verbal (telephonic) approval will be obtained from the Sponsor followed by written documentation by BNW to the Sponsor. Any change which impacts the cost or schedule of the study will be documented in writing and approved by the Sponsor Representative's signature prior to implementation. The Sponsor may initiate modification to the protocol by telephonic authorization to BNW followed by written documentation as stated above. This study will be conducted in compliance with Good Laboratory Practice

[1]This procedure is currently in use by the National Toxicology Program, NIEHS, Research Triangle Park, NC.

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regulations, 21 CFR 58. All records required to reconstruct the study will be maintained as stipulated in 21 CFR 58.190. All paraffin blocks and wet tissues resulting from any portion of this study will be retained by BNW until 6 months after acceptance of the final report at which time they will be sent to the Sponsor. If requested by the sponsor, all microscopic slides will be submitted to the Sponsor with the final draft report. Records to be retained in study archives will be specified by the SOP entitled "Records to be Retained for Philip Morris Studies". Records that accumulate during the study will be retained at BNW until they are shipped to the Sponsor immediately prior to the termination of the contract or until the Sponsor requests transfer, whichever occurs first.

F. Draft Report

Following a Quality Assurance audit of the raw data and data tables, a complete and detailed draft report will be submitted to the Sponsor within the time frame of the contract.

G. Reference

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XXIII. BNW APPROVAL

Earl W. Morgan, DVM, ACVPM, DABT
Principal Investigator

Date

R.A. Gelman, MS
Quality Assurance Auditor

Date

XXIV. SPONSOR APPROVAL

George J. Patskan, PhD
Sponsor's Representative

Date

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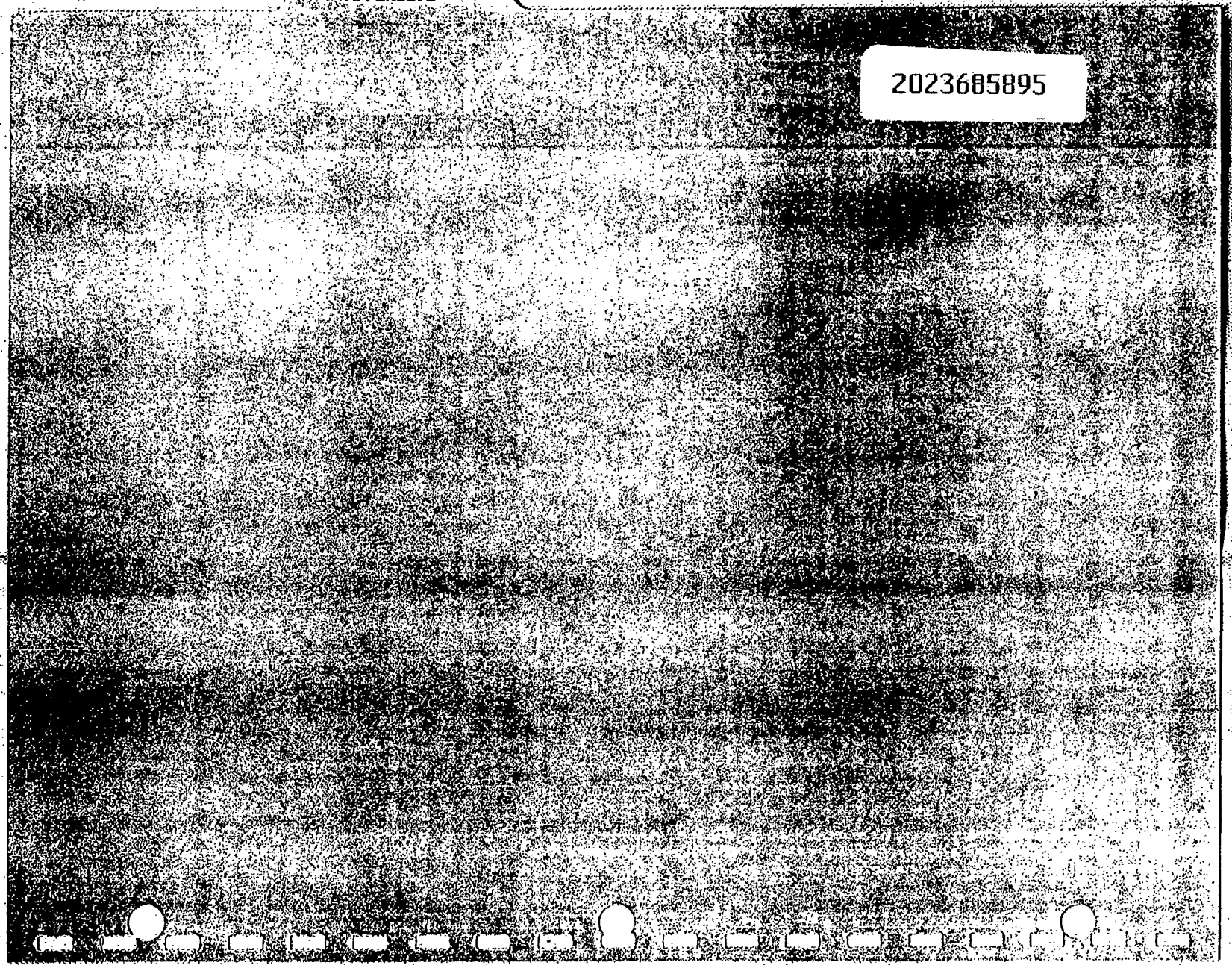
APPENDIX B



APPENDIX B

RESUMES FOR KEY PERSONNEL

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APPENDIX B

RESUMES FOR KEY PERSONNEL

2023685897

"Use or disclosure of proposal data is subject to the restriction on the Title page of this Proposal."

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BILLY J. CHOU

Staff Scientist, Technical Group Leader, Inhalation Toxicology Technical Group, Toxicology Department, Battelle, Pacific Northwest Laboratories

B. Vet. Sci. (1964), National Taiwan University

D.V.M. (1964), National Taiwan University

M.S. Animal Nutrition (1968), Kansas State University

Ph.D. Physiology (1972), Kansas State University

Diplomate of the American Board of Toxicology (1981).

Dr. Chou joined Battelle in 1984 as Principal Investigator/Toxicologist for the NTP Program. His responsibilities are:

- To be knowledgeable and up to date in all aspects of the program, including the status of toxicity test(s), recordkeeping, reporting and detailed analysis of cost for any or all functions that make up the total operation
- To be familiar with protocols to be included in studies for a particular chemical, observe animals for clinical signs and recommend dose levels for each test phase.

Previously, he was employed as Head of the Toxicology and Animal Facility, Department of Pharmacology and Toxicology, William H. Rorer, Inc. (1976-1984) and, in the same department, Senior Pharmacologist (1973-1975). His experience has included:

- Design, supervision, evaluation and reporting of toxicity and target organ toxicity (mechanism) studies involving various chemical and potential therapeutic agents in mice, rats, guinea pigs, rabbits, dogs, nonhuman primates and pigs. Routes of administering drugs included oral, intramuscular, intravenous, dermal, ocular and vaginal.
- Design and evaluation of a battery of short-term tests for detecting gene mutations, chromosome aberrations and primary DNA damage.

In his assignment as Head of Toxicology and Animal Facility, Dr. Chou supervised animal husbandry for mice, rats, guinea pigs, rabbits, miniature pigs, dogs and rhesus monkeys. He instituted standard procedures in accordance with the Animal Welfare Act of 1970 and HEW Publication (NIH) 74-23, "Guide for the Care and Use of Laboratory Animals." Under his guidance the facility was accredited by the American Association for Accreditation of Laboratory Animal Care, and was inspected by both the U.S. Department of Agriculture and, for Good Laboratory Practices, by the U.S. Food and Drug Administration.

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Publications

Chou, B. J., C. J. Chang, and Y. C. Lee. 1965. The study of climatical effect on parasites on goat. J. Taiwan Assoc. Anim. Husb. Vet. Med. 10:26-27.

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JOHN R. DECKER

Research Scientist, Technical Group Leader, Bioengineering and Exposure Technology Technical Group, Toxicology Department, Battelle, Pacific Northwest Laboratories

B.S. Electrical Engineering (1967), Colorado State University

Physiology (1968), Colorado State University (graduate study)

Physiology (1976), Washington State University (graduate study)

Mr. Decker joined the group in 1969, and has developed a bioengineering capability within the department. He is presently Supervisor of the Toxicology Bioengineering Group.

Mr. Decker has participated in many areas of study, including biological effects of chemical aerosols, vapors and gases, biological effects of magnetic and electric fields, effects of cigarette smoke, effects of inhaled radionuclides, effects of fly ash and sulfuric acid, electrical stimulation of bone growth into porous material, effects of endogenous heat from implanted electrical and nuclear-powered sources, detection of "bends" by Doppler flow metering, detection of epilepsy in dogs by EEG analysis, and improved methods of male and female fertility control. Specific contributions include design and fabrication of a liquid vapor generation system, a cigarette smoke exposure system, a minicomputer-controlled radioisotope aerosol exposure system, equipment for a general lung-function laboratory, both magnetic field and electromagnetic radiation exposure systems, specialized electrocoagulation instruments for sterilization of both male and female reproduction systems, and a condom field testing system.

Mr. Decker has designed and constructed fully automated, computerized data acquisition and control systems for a 90-chamber inhalation toxicology laboratory and for a wind tunnel obscurant test system.

Mr. Decker is Assistant Principal Investigator for both Exposure and Sampling System Setup and Animal Exposures for NTP-sponsored inhalation exposure projects.

Before joining the Biology and Chemistry Department, Mr. Decker worked for 1 year in the Occupational and Environmental Protection Department, designing and testing radiation detection and dosimetry instrumentation.

As an Electronics Engineer in the Department of Physiology at Colorado State University (1968), Mr. Decker's graduate work included the design and construction of biomedical instrumentation for research in subprimate neurology. During his undergraduate years Mr. Decker was also employed as an Electronics Technician at Honeywell, Denver, CO (1966-1967).

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B.S. Chemistry (1979), University of New Mexico

Ph.D. Analytical Chemistry (1983), Colorado State University

Dr. Dill joined the Department in 1987. His duties involve development and evaluation of analytical methodologies used in chemical characterizations and qualification of test atmospheres used in inhalation toxicology studies. He is currently working with formic acid and glutaraldehyde exposures.

Previously, Dr. Dill was employed by Pacific Northwest Laboratory (PNL) in waste packaging studies for nuclear waste repository investigations. Before joining PNL, he worked for Rockwell Hanford Operations as a Solution Chemistry Laboratory Team Leader. His experience was principally in inorganic analysis of natural water samples, supporting nuclear waste repository investigations.

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R: REDACTED MATERIAL

R. ANN GELMAN

Quality Assurance Specialist, Quality Assurance Unit, Life Sciences Center, Battelle, Pacific Northwest Laboratories

B.S. Foods and Nutrition (1960), Carnegie Mellon University

M.S. Nutrition (1965), University of Pittsburgh

A.A. Quality Assurance (1985), Columbia Basin College

Ms. Gelman, who has been with Battelle since 1976, was assigned to the Quality Assurance Unit (QAU) in February 1981. She is responsible for supervising the activities of the QAU; for assuring management that projects conducted under Good Laboratory Practices (GLP) regulations for nonclinical laboratory studies are in compliance with those regulations; for assuring management that the research facility is in compliance with those regulations; for audit and surveillance activities necessary to assure compliance; for working with Project Managers (Study Directors) in setting up GLP procedures and documentation for their projects (studies); for coordinating activities during external audits by government and client representatives.

Previously (1976-1981), Ms. Gelman was a Technical Specialist in the Cell Biology Section of the Biology and Chemistry Department. She was responsible for working with staff scientists in various areas, including the toxicology of inhaled fossil-fuel-related carcinogens and of inhaled polyaminocarboxylic acids used for excorporation of radionuclides.

Before coming to Battelle, Ms. Gelman was a Senior Research Assistant in the Department of Pharmacology and Physiology, School of Dental Medicine, University of Pittsburgh (1968-1976). Her responsibilities included supervising a research laboratory which specialized in the biochemistry of trace lipids.

Prior to that time, Ms. Gelman was Senior Research Dietitian at the Clinical Research Center, Stanford Medical Center, Palo Alto, CA (1966-1968). She supervised the activities of the Dietary Department of the Center.

From 1960-1964 Ms. Gelman was Research Dietitian, Department of Biochemistry and Nutrition, Graduate School of Health, University of Pittsburgh. She planned and implemented research diets, and instructed patients regarding therapeutic diets.

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R: REDACTED MATERIAL

ANDREW W. GIESCHEN

Scientist, Analytical Chemistry Technical Group, Toxicology Department, Battelle, Pacific Northwest Laboratories

B.S. Chemistry (1983), Arizona State University

B.S. Microbiology (1983), Arizona State University

M.S. Chemistry (1986), Washington State University

Mr. Gieschen is currently enrolled in the Tri-Cities University Center Master's Degree program for Electrical Engineering.

Mr. Gieschen joined the section in 1987. He is involved in measurement of chemicals in support of inhalation toxicology programs. His responsibilities include preparing reports and proposals and developing experimental methods and protocols. Current projects on which he is working include inhalation exposures to chloroprene, isoprene, butadiene, HCCPD, formic acid, tetrahydrofuran, acetonitrile and glutaraldehyde.

As a Graduate Research Assistant at Washington State University (1984-1986), Mr. Gieschen performed research on the synthesis and characterization of organic compounds used to study hydrogen peroxide solvylitic rearrangements, including computer modeling of macrocycles. He has a strong interdisciplinary background in biochemical, pharmaceutical, and organic chemistry, with academic training in electrical engineering and computer programming. He has experience with flash chromatography and thin-layer chromatography, has operated the Cary UV-VIS spectrophotometer, Jeol FX 90Q NMR, Nicolet R3m/E diffractometer, Nicolet MX-90, FT-IR, and other common chemistry techniques and instruments.

He has also had extended experience with personal computers (DOS, dBASE II & III, LOTUS 1,2,3, Fortran, Pascal, BASIC, Assembly Language, Word, WordStar) and the CMS operating system.

As an undergraduate Research Assistant at Arizona State University (1982-1983), Mr. Gieschen investigated the role of tannins in the defense of plants, gaining experience in visual spectrophotometry, experiment design, and computer data management using PDP-II, Wylbur, SAS, and Minitab.

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R: REDACTED MATERIAL

BERNARD J. GREENSPAN

Staff Scientist, Technical Group Leader, Aerosol and Inhalation Research (AIR) Technical Group, Toxicology Department, Battelle, Pacific Northwest Laboratories

B.A.	Physics (1975), Clark University
M.S.	Biophysics (1978), University of Rochester Medical Center
Ph.D.	Biophysics (1981), University of Rochester Medical Center

Dr. Greenspan joined the Department in 1985. He is responsible for designing inhalation toxicity studies, basic aerosol research, and exposure task management for NTP and industrial clients.

Previously (1983 to 1985), he was a Postdoctoral Participant in the Radiobiology Group of the Lovelace Inhalation Toxicology Research Institute, where he was involved in studies of the toxicity of lithium combustion aerosols and of the metabolism of inhaled beryllium aerosols. From 1981 to 1983 he was a Staff Scientist in the Inhalation Department of the Union Carbide-Mellon Institute, Bushy Run Research Center. He was responsible for the design of aerosol, dust and vapor exposure systems. From 1973 to 1975 Dr. Greenspan was employed as a Technical Specialist at the University of Massachusetts Medical School. He was involved in studies on the knee-joint afferent neurons in cats and monkeys, and in examining the effects of pharmacological agents on transmission across the neuromuscular junction of rat diaphragms.

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TERRYL J. MAST

Staff Scientist, Inhalation Toxicology Technical Group, Toxicology Department, Battelle, Pacific Northwest Laboratories

B.S. Biochemistry/Biophysics (1974), University of California, Davis

M.S. Agricultural Chemistry (1979), University of California, Davis

Ph.D. Environmental Chemistry (1983), Department of Environmental Toxicology, University of California, Davis

Postdoctoral Appointment, Developmental and Reproductive Biology (1984-1985), California Primate Research Center, University of California, Davis

Member, Graduate Faculty, Department of Toxicology, Oregon State University, Corvallis, OR

Member, Graduate Faculty, Graduate Program in Pharmacology/Toxicology, Washington State University, Pullman, WA

Diplomate, American Board of Toxicology

Dr. Mast joined the department in 1986. Her research interests are in the area of reproductive and developmental toxicology, with emphasis on teratology, mechanisms of developmental toxicity and pharmacokinetics associated with teratologic studies. She is currently Project Manager of the NTP-sponsored Inhalation Reproductive Toxicology Program assessing the potential reproductive toxicity of industrial solvents and intermediates, and aerosols of compounds such as cadmium oxide and gallium arsenide. As Project Manager, Dr. Mast reviews the toxicological literature on the test agent, proposes the study design, and performs the developmental toxicity studies.

Dr. Mast is studying mechanisms of the developmental toxicity induced by complex mixtures in rodents and is working on an *in vitro* system to study the ontogeny of the toxic effects in the developing lung. Lung hypoplasia produced during organogenesis, after exposure to certain fractions of complex mixtures is of special interest.

Dr. Mast joined the department following 2 years of work in developmental toxicology at the California Primate Research Center, where she studied the teratology, pharmacokinetics, and placental transfer of valproic acid in nonhuman primates and rodents. Her interest in developmental metabolism and maternal-fetal pharmacokinetics led to studies on the development of hepatic xenobiotic-metabolizing enzymes in the fetal monkey. She conducted a study in rats to correlate teratological effects of the pesticide oftanol with maternal plasma kinetics, cholinesterase activity and metabolism during organogenesis. She also participated in a study that correlated plasma levels and toxic effects on the male reproductive system of dibromodichloropropane (DBCP) administered to rhesus monkeys in drinking water.

Her 6-year tenure as Lecturer in the Environmental Toxicology Department at the University of California, Davis gave her an extensive background in the occurrence and effects of environmental toxicants. During this time she taught lecture and laboratory courses in environmental and analytical toxicology to graduates and undergraduates. Her doctoral thesis work, also performed in that department, concentrated on the collection, separation, identification and mutagenicity of airborne complex mixtures produced as a result of agricultural burning.

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PAUL W. MELLICK

Staff Pathologist, Pathology Technical Group, Toxicology Department, Battelle, Pacific Northwest Laboratories

D.V.M. Veterinary Medicine (1962), The Ohio State University

M.S. Veterinary Pathology (1964), Cornell University

Ph.D. Comparative Pathology (1975), University of California, Davis

Dr. Mellick joined Battelle in 1987. As a staff pathologist, his duties include supervising necropsy and providing gross and microscopic pathology descriptions, documentation, data analysis and interpreting lesions in laboratory animals used in toxicology studies. He has conducted pathology evaluations for 14-day repeated-dose inhalation studies of furfuryl alcohol, nitromethane, gallium arsenide, 2-chloronitrobenzene, and 4-chloronitrobenzene; 28-day inhalation studies of ozone; 90-day subchronic inhalation studies of furfuryl alcohol, gallium arsenide, 2-chloronitrobenzene, cadmium oxide, glutaraldehyde, and isoprene; 90-day gavage studies of lewisite; 6-month inhalation toxicity studies of isoprene, and 24-month inhalation carcinogenicity studies of tetrafluoroethane. Studies listed above were conducted in either rats or mice, or both, and all except the lewisite gavage study were sponsored by the National Toxicology Program.

Dr. Mellick serves as Principal Investigator for nose-only inhalation toxicity and carcinogenicity studies of a pharmaceutical agent sponsored by a prominent pharmaceutical company. In this capacity, he has overall project management responsibility; develops proposals and study protocols; coordinates efforts of senior staff members in the disciplines of chemistry, aerosol technology, bioengineering, toxicology, animal care, pathology, health and safety, and quality assurance to ensure that all requirements of the studies are met; monitors and controls expenditures of project funds; and communicates with the sponsor concerning progress, technical concerns, and potential problems.

Dr. Mellick also served as the Principal Investigator for the NTP/HEI Collaborative Study on the Effects of Chronic Ozone Exposure. In addition to overall program management responsibility, he coordinated the efforts of ten groups of investigators from different laboratories with the Battelle staff in ten different studies designed to evaluate the toxic effects (other than carcinogenesis) of exposure of rats to ozone for 20 months.

He received Board Certification in Veterinary Pathology from the American College of Veterinary Pathologists in 1970.

Dr. Mellick has completed over 21 years of active duty in the United States Army Veterinary Corps, retiring at the rank of Colonel. All assignments involved U.S. Army medical research and development activities. All positions since July 1966 were active duty military assignments.

While Deputy Commander at Letterman Army Institute of Research, Presidio of San Francisco, CA from July 1986 to October 1987, he planned, directed and managed scientifically diverse biomedical research programs with a staff of 250 and an annual budget of $14 million. He directly supervised chiefs of seven research divisions and provided second-level supervision for 20 civilian and military scientists. He also served as Pathologist-in-Charge of Good Laboratory Practices (GLP)-compliant toxicology studies.

Dr. Mellick was Chief, Division of Comparative Medicine and Toxicology, at Letterman Army Institute of Research from August 1984 to June 1986. He planned and managed research and research support activities in toxicology, pathology, and analytical chemistry, experimental surgery, and laboratory animal

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care. He also conducted GLP-compliant toxicology studies; designed and implemented multidisciplinary studies on effects of chemical warfare agents.

From March 1983 to August 1984, he was Head, Department of Veterinary Medicine, U.S. Naval Medical Research Unit, Cairo, Egypt. While there, he planned, directed and conducted research in infectious zoonotic diseases in Egypt and other Middle Eastern countries.

As Chief of the Pathology Service at Letterman Army Institute of Research from August 1976 to March 1983, Dr. Mellick conducted pathology evaluations on laboratory animals used in studies of toxicology, effects of blood replacement solutions, nutrition and experimental surgery. He organized and developed GLP-compliant toxicology research and testing capability. He also conducted a residency training program in Veterinary Pathology which prepared eight officers for successful completion of the Board Certification Examination of the American College of Veterinary Pathologists.

Dr. Mellick was Chief, Electron Microscopy Division at Letterman Army Institute of Research from July 1975 to August 1976. He conducted and directed research, service and training functions of the Electron Microscopy Division. His duties were incorporated into those of Chief, Pathology Service, described in the preceding paragraph.

He was a graduate student in Comparative Pathology, Department of Pathology, School of Veterinary Medicine, University of California, Davis, CA from August 1972 to June 1975. During that time, he studied mechanisms and pathogenesis of pulmonary injury caused by inhalation of air pollutants. This research was the basis of his Ph.D. dissertation.

From July 1970 to August 1972, he was Chief, Toxicology Division, U.S. Army Environmental Hygiene Agency, Edgewood Arsenal, MD. He conducted toxicology studies on insecticides, insect repellents, hydrogen sulfide gas, and fluorocarbons. These studies included acute, subacute and subchronic oral, dermal, ocular, and inhalation toxicology protocols and tests for teratogenesis and behavioral effects.

As a resident in Veterinary Pathology at the Armed Forces Institute of Pathology at Walter Reed Army Medical Center, Washington, DC from November 1968 to July 1970, he performed histologic evaluation of tissues from animals used to determine the safety of irradiated foods. He prepared case reports on pathology materials submitted for consultation and participated in the Veterinary Pathology training program. This training was verified by his success in the Board Certification Examination of the American College of Veterinary Pathologists.

Dr. Mellick was a Veterinary Pathologist at the National Institute of Bacteriology and Pathology, Saigon, Republic of Vietnam from October 1967 to October 1968. At that time, he conducted safety and efficacy studies on modified live-virus vaccines and bacterins for swine and poultry. He also established an animal disease diagnostic laboratory.

From August 1966 to October 1967, he was a trainee in Veterinary Pathology at the Armed Forces Institute of Pathology, Walter Reed Army Medical Center, Washington, DC.

He was a General Practitioner from June 1964 to July 1966 at the Champlain Veterinary Clinic, St. Albans, VT where he practiced clinical veterinary medicine and surgery in large and small animals.

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RODNEY A. MILLER

Staff Pathologist, Pathology Technical Group, Toxicology Department, Battelle, Pacific Northwest Laboratories

D.V.M. Veterinary Medicine (1971), Iowa State University, with distinction

Ph.D. Veterinary Pathology (August 1979), Washington State University

Dr. Miller has been with the Department as a Research Pathologist since July 1979. He has been involved in the histopathologic evaluation of rats exposed to acid aerosols and in evaluation of histologic sections of lungs, kidneys, livers, ovaries, and uteruses of rats and rabbits exposed to numerous inhaled chemicals in conjunction with teratologic studies. Dr. Miller is assigned to necropsy supervision and numerous repeated-dose, subchronic and chronic histopathology portions of the National Toxicology Program on carcinogenesis and toxicity in rodents. He has evaluated, in rats, the long-term effect of inhaled americium nitrate, and the mammary carcinogenicity of injected plutonium citrate. He has also evaluated rats and guinea pigs that inhaled solvent-refined-coal (SRC) process solvent (short-term), and rats that inhaled diesel exhaust and coal dust (short-term) after treatment (or no treatment) with porcine pancreatic elastase. Dr. Miller has examined the respiratory tracts of rats acutely exposed to fog oils and red phosphorus smoke, and has examined fetal rat lungs in which the maternal rat had been exposed to SRC products. Dr. Miller has also examined the gills of rainbow trout after the gills were exposed to volcanic ash. Dr. Miller has performed histopathologic evaluations of rat livers for induction of gamma glutamyl transpeptidase foci after exposure to SRC products. He has performed comprehensive histopathology of rats and mice exposed subchronically and chronically to a heavy distillate from the SRC-II process. He has performed limited transmission electron microscopy of the livers of the heavy-distillate-exposed animals.

Dr. Miller has been involved in necropsy and disease diagnosis of the Hanford Miniature swine breeding herd.

After practicing small animal medicine (1971-1975), Dr. Miller was a postdoctoral Research Associate, Department of Veterinary Microbiology and Pathology, Washington State University (1975-1979). Besides instructing undergraduates in Pathology, he has had 4 years of experience in morphologic/diagnostic pathology at the Washington Animal Disease Diagnostic Laboratory, Washington State University. Much of the diagnostic work at Washington State University involved tumor diagnosis in domestic animals. His Ph.D. research concerned the interrelationship of experimental atherosclerosis and alloxan diabetes in the rabbit.

Dr. Miller is a Diplomate of the American College of Veterinary Pathologists (September 1980). He is also a member of the Society of Toxicologic Pathologists.

Dr. Miller has been licensed to practice veterinary medicine in Washington since 1971. He was a member of the Iowa State University Honors Program.

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HARVEY A. RAGAN

Manager, Toxicology Department, Life Sciences Center, Battelle, Pacific Northwest Laboratories

B.S. Biology (1956), Washington State University

D.V.M. Veterinary Medicine (1959), Washington State University

Special Fellow, NIH (1967-1969), Hematology, University of Utah

Diplomate (1987), American Board of Toxicology

Dr. Ragan joined the Department as a Research Scientist in 1962 after 3 years in private practice. His research interests for the past 20 years have been concerned with the broad aspects of hematopoiesis and lymphopoiesis. Studies have been directed to alterations in these systems following insults by radionuclides, heavy metals, and physical agents. Specific investigations include radiotracer kinetics of the cellular elements of blood, iron metabolism, reticuloendothelial functions, carcinogenesis, and evaluation of immune competence. Dr. Ragan has been Principal Investigator for DOE-funded programs on the study of interrelationships between pollutant metals and nutritional deficiencies on the hematopoietic and immune systems, the clinical pathologic effects of electric fields on rodents, and the in vitro quantitation of effects of pollutants on hematopoietic stem cells; a program for EPRI on the hematologic effects of electric field exposures; and a U.S. Navy program on the hematoimmunologic effects of exposure to pulsed microwaves.

In addition to his research interests, Dr. Ragan directs the Clinical Pathology Laboratory and provides consultation and collaboration for this discipline. As a research support group, this well-equipped laboratory provides determinations of hematologic, coagulation, serum chemistry and urine parameters for most laboratory animal species. In addition, special hematologic determinations, cytochemistry and bone marrow evaluations are developed and performed as needed for specific studies.

Responsibility as Manager of Toxicology Department includes administrative control of an annual budget of >$10 million and about 16 scientists, 26 technical specialists, 25 technicians, and 10 secretaries/clerks.

For a 2-year period starting in January 1984, Dr. Ragan was Principal Investigator on multimillion dollar toxicity and carcinogenesis studies conducted by Battelle for the National Toxicology Program.

Dr. Ragan was an NIH Special Fellow in hematology under the sponsorship of Dr. George E. Cartwright at the University of Utah Medical School during 1967-1969. His research involved the interrelationships between iron and copper on erythropoiesis in young swine.

Prior to the fellowship, Dr. Ragan's research at Hanford involved clinical and toxicologic studies into the effects of radionuclides in miniature swine, miniature goats, and sheep. He was also involved in a National Heart, Lung, and Blood Institute-funded program on the chronic effects of added endogenous heat in miniature swine implanted with a heat source in the thoracic aorta.

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Dr. Ragan is licensed to practice Veterinary Medicine, Surgery, and Dentistry in Washington, and has Federal accreditation in Washington.

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ROGER A. RENNE

Staff Pathologist, Technical Group Leader, Pathology Technical Group, Toxicology Department, Battelle, Pacific Northwest Laboratories

Pre-Veterinary (1962), Portland State University

D.V.M. Veterinary Medicine (1966), Washington State University

Diplomate of the American College of Veterinary Pathologists (1973)

From 1978 to the present, Dr. Renne has served as Task Leader for all pathology tasks on the NTP inhalation carcinogenesis project. He has been a participant in a number of pathology working groups for NTP-sponsored subchronic and chronic inhalation studies performed at Battelle and has participated in a number of peer reviews of pathology data from rodent inhalation carcinogenesis and toxicology studies for industrial sponsors.

From 1985 to the present, Dr. Renne has served as Pathology Task Leader for several nose-only inhalation studies of humectants and cigarette smoke in rats and hamsters for a private industrial sponsor.

From 1988 to 1990, Dr. Renne served as the Principal Investigator and Pathology Task Leader for a confidential industrial sponsor on an inhalation study investigating the toxic effects of inhaled liquid aerosols in rats.

From 1978 to 1986, Dr. Renne was Principal Investigator for a DOE-sponsored project that assayed the carcinogenic potential of crude materials, products, and chemical and boiling-point fractions of these materials from several synthetic fossil fuel technologies, utilizing chronic (2-year) skin-painting exposure of mice.

From November 1979 to April 1982, Dr. Renne was Principal Investigator on a NIOSH-sponsored project studying the long-term fibrogenic potential of four particulate materials intratracheally instilled in hamsters.

From August 1979 to November 1980, Dr. Renne was Principal Investigator of an inhalation carcinogenesis project which involved the bioassay of 15 environmental chemicals in rodents, performed at Battelle as part of the Carcinogenesis Bioassay Program of the National Cancer Institute (NTP), under Prime Contract to Tracor Jitco, Inc.

Shortly after joining the Department in 1976, Dr. Renne was Principal Investigator on several projects, including studies evaluating the fibrogenic and carcinogenic potential of particulate oil shale materials using repeated intratracheal instillation, and inhalation toxicity studies of H_2S and ammonia in rodents. He was also responsible for histopathologic evaluation of pulmonary lesions in dogs exposed to cigarette smoke, and gross and microscopic evaluation of tissues from dogs exposed to carcinogenic doses of particulate radionuclides via inhalation.

While on the staff of Experimental Pathology Laboratories (1974-1976), Dr. Renne served as a Consultant Pathologist to the Tumor Pathology Branch of the National Cancer Institute, Bethesda, MD. Specific duties included histopathologic evaluation of tissues from rodents exposed to pesticides and other environmental chemicals as part of the NCI Chemical Carcinogenesis Bioassay Program, and consultation in revising of the Systematized Nomenclature of Pathology for use in the NCI Carcinogenesis Bioassay Program.

During military service (1968-1974) Dr. Renne was a Veterinary Pathologist, Biomedical Laboratory, Edgewood Arsenal, MD (1973-1974). Dr. Renne participated in research programs determining the

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toxicity, pharmacology, and pathology of chemical compounds of military interest. He performed necropsies on laboratory animals that had been exposed to chemical compounds. He conducted detailed microscopic pathology studies in order to assess the nature and degree of histologic responses to chemical compounds or to determine the cause of death in colony animals dying spontaneously. Specific investigations of laboratory animal diseases included glomerulonephritis in laboratory rabbits; measles-virus-induced abortion and endometritis in rhesus monkeys, and tuberculosis in rhesus monkeys. He prepared reports describing and interpreting gross and microscopic alterations occurring in these animals. He conducted a formal training program in veterinary pathology acceptable to the American College of Veterinary Pathologists.

Prior to this (1970-1973), Dr. Renne was Resident in Veterinary Pathology at the same laboratory. His activities involved gross and microscopic pathology and toxicology of chemical compounds of military interest and spontaneous laboratory animal diseases. He was a Preceptee in the U.S. Army preceptorship program in Veterinary Pathology.

Dr. Renne was assigned as Veterinary Officer, Toxicology Division, U.S. Army Environmental Hygiene Agency at Edgewood Arsenal from 1968 to 1970. His responsibilities included design and conduct of research and toxicologic evaluation of chemical compounds of military interest, including gross and microscopic pathologic examination. He was also responsible for the care of a colony of laboratory animals.

From 1966 to 1968 Dr. Renne was in private veterinary practice in Albany, OR.

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STEPHEN E. ROWE

Staff Veterinarian, Life Sciences Center, Battelle, Pacific Northwest Laboratories

B.S. Agriculture (Biochemistry option; 1964), Purdue University

D.V.M. Veterinary Medicine (1968), Purdue University

M.S. Pathology (1972), University of Michigan

Diplomate of the American College of Laboratory Animal Medicine (1973)

Dr. Rowe directs the Animal Health Program for the Life Sciences Center. He is responsible for development of appropriate procedures for the acquisition and maintenance of pathogen-free animals and for minimizing environmental variables. He evaluates the health of animals upon their arrival and monitors the health and environment of animals on study. Dr. Rowe utilizes clinical and histopathological examinations and the support of the Clinical Microbiology Laboratory, which he directs, to carry out these responsibilities.

As Staff Veterinarian for ongoing projects, Dr. Rowe:

- Develops and maintains diagnostic services and disease surveillance procedures
- Develops and implements a preventive medicine program and related standard operating procedures
- Provides veterinary care, including surgery, postsurgical care, and physical examinations
- Performs gross necropsy and histopathological examinations of animals in diagnosing spontaneous diseases
- Trains technical staff in procedures involving animals
- Monitors feed, water and bedding quality
- Monitors sanitation and environmental conditions in the animal facilities
- Selects or approves sources of animals and conducts pathogen screening procedures on all shipments of animals
- Provides expertise on animal care, animal health, and humane procedures in planning experiments and preparing proposals
- Evaluates experimental effects in live animals and aids in the interpretation of experimental results
- Chairs the Animal Resources Council, which is charged with planning, communicating and facilitating animal care policies and procedures in the Life Sciences Center.

Dr. Rowe also serves as the "institutional veterinarian" and is Chairman of the Animal Care Committee. As such, he is responsible for assuring Battelle's compliance with the USDA Animal Welfare Regulations, the NIH Animal Welfare Policy and other applicable regulations and guidelines concerning animal care and use. In carrying out these responsibilities (or in association with the Animal Care Committee), he:

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- Monitors animal care and use on a daily basis
- Reviews all Battelle proposals involving the use of vertebrate animals
- Reviews all ongoing research projects using animals annually
- Conducts semiannual inspections and reviews of the animal facilities and of the Animal Care and Use Program
- Provides education and training in animal welfare and in animal care and use procedures for all staff working with animals
- Provides guidelines and training in the use of analgesics and anesthetics and their appropriate application for pain control and euthanasia

Dr. Rowe was a Postdoctoral Fellow with the Unit for Laboratory Animal Medicine at the University of Michigan Medical School from 1970-1973. During this time, and for a period of more than 1 year, Dr. Rowe had first-line responsibility for the clinical veterinary care of several hundred primates and of a diverse population of other animals used at the University of Michigan Medical School. Training also included extensive practical experience with the gross and histopathological diagnosis of diseases in laboratory animals.

From 1968 to 1970, Dr. Rowe was a Veterinary Corps Officer with the Army Environmental Hygiene Agency, Edgewood Arsenal, MD. He conducted primary irritation, sensitization and other toxicity studies in laboratory animals and directed the animal care activities of that facility.

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R. BRUCE WESTERBERG

Staff Scientist, Technical Group Leader, Analytical Chemistry Technical Group, Toxicology Department, Battelle, Pacific Northwest Laboratories

B.S. Chemistry (1969), Southern Oregon State College

Ph.D. Analytical Chemistry (1974), Purdue University

Dr. Westerberg joined the Department in 1980. He provides chemistry support for inhalation studies, and is presently a Chemistry Task Leader for inhalation toxicology studies. He is also Technical Group Leader for the Analytical Chemistry Group. The responsibilities of this work include development of on-line methods for monitoring exposure levels, their implementation, and studies of the possible degradation of exposure levels. Dr. Westerberg has been the Principal Investigator for several projects involving determination of trace levels of a test material and major metabolites in plasma. He has also provided chemistry support for studies of the metabolism of carcinogens in the presence of complex organic mixtures.

Dr. Westerberg has also assisted in the operation of a high-resolution mass spectrometer; this involves analyses requiring a variety of techniques: determination of elemental composition by exact mass measurements, trace analysis using fused silica column gas chromatography and selected ion monitoring, field desorption, negative ion chemical ionization, etc.

Previously (1974-1980), Dr. Westerberg was employed by E. I. duPont Chambers Works, Jackson Laboratory, as an Analytical Research Chemist. During part of that period he was responsible for the laboratory's HPLC facilities. He also upgraded the GC/MS facilities and demonstrated increased analytical capability provided by new instrumentation, with emphasis on quantitative trace analysis. This work included analysis of industrial waste waters for priority pollutants, and analysis for polychlorinated biphenyls (PCBs) in complex materials.

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Wehner, A. P., R. A. Renne, B. J. Greenspan, H. S. DeFord, H. A. Ragan, R. B. Westerberg, C. W. Wright, R. L. Buschbom, G. T. Burger, A. W. Hayes, C. R. E. Coggins, and A. T. Mosberg. 1990. Comparative subchronic inhalation bioassay in hamsters of a cigarette that only heats tobacco. Inhal. Toxicol. 2:255-284.

Gieschen, A. W., B. J. Greenspan, R. B. Westerberg, T. J. Goehl, and J. H. Roycroft. 1991. Generation, monitoring, and concentration verification of PPB concentrations of glutaraldehyde for inhalation studies. The Toxicologist 11:105 (abstract).

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Mast, T. J., R. J. Weigel, R. B. Westerberg, B. A. Schwetz, and R. E. Morrissey. Evaluation of the potential for developmental toxicity in rats and mice following inhalation exposure to tetrahydrofuran. Fundam. Appl. Toxicol. (in press).

Renne, R. A., A. P. Wehner, B. J. Greenspan, H. S. DeFord, H. A. Ragan, R. B. Westerberg, R. L. Buschbom, G. T. Burger, A. W. Hayes, R. L. Suber, and A. T. Mosberg. Two-week and thirteen-week inhalation studies of aerosolized glycerol in rats. Inhal. Toxicol. (in press).

Presentations

Rogers, L. B., A. T. Shepard, R. B. Westerberg, et al. 1976. New Studies in High Precision Gas Chromatography. Presented at the 27th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, 1976.

Kelman, B. J., D. L. Springer, and R. B. Westerberg. 1983. Metabolic Distribution of Benzo(a)pyrene (BaP) in the Circulation of the Rodent. Presented at the Society of Toxicology Meeting, March 7-11, 1983, Las Vegas, NV.

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Dill, J. A., R. B. Westerberg, L. A. Pingel, and W. C. Forsythe. 1988. Automated Determination of Formic Acid Vapors using Long Pathlength Infrared Spectroscopy. Presented at the 43rd American Chemical Society Northwest Regional Meetings, June 29-July 1, 1988, Spokane, WA.

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Lee, R. N., R. B. Westerberg, D. R. Baer, and M. H. Engelhard. 1988. Application of Particle-Induced X-Ray Emission to Inhalation Toxicology Studies. Presented at the 43rd American Chemical Society Northwest Regional Meetings, June 29-July 1, 1988, Spokane, WA.

Renne, R. A., A. P. Wehner, B. J. Greenspan, R. B. Westerberg, et al. 1988. Subchronic Inhalation Study in Hamsters, Comparing Smoke from a Cigarette Which Burns and One That Only Heats Tobacco. Presented at the Society of Toxicology Meeting, February 15-19, 1988, Dallas, TX.

Wehner, A. P., R. A. Renne, B. J. Greenspan, H. S. DeFord, H. A. Ragan, R. B. Westerberg, C. W. Wright, and O. R. Moss. 1988. Subchronic Inhalation Study in Rats, Comparing Cigarette Emissions from Combusted and Non-Combusted Tobacco. Presented at the Society of Toxicology Meeting, February 15-19, 1988, Dallas, TX.

Westerberg, R. B., J. R. Decker, and T. J. Goehl. 1988. Analytical Chemistry Support of the National Toxicology Inhalation Studies Performed at Battelle-Northwest. Presented at the 43rd American Chemical Society Northwest Regional Meetings, June 29-July 1, 1988, Spokane, WA.

Westerberg, R. B. 1989. Sensitivity of Monitoring Methods. Presented at the NTP Chemistry and Inhalation Workshop, January 17-19, 1989, Research Triangle Park, NC.

Gieschen, A. W., B. J. Greenspan, R. B. Westerberg, T. J. Goehl, and J. H. Roycroft. 1991. Generation, Monitoring, and Concentration Verification of PPB Concentrations of Glutaraldehyde for Inhalation Studies. Presented at the 30th Annual Meeting of the Society of Toxicology, February 25-March 1, 1991, Dallas, TX.

Weigel, R. J., M. L. Clark, R. B. Westerberg, J. R. Decker, and T. J. Goehl. 1991. Use of a Rotary Evaporator System to Generate High Purity Vapors from Reactive Liquids for Inhalation Studies. Presented at the 30th Annual Meeting of the Society of Toxicology, February 25-March 1, 1991, Dallas, TX.

Patent

Burton, F. G., R. E. Schirmer, and R. B. Westerberg. 1982. Positive End-of-Service-Life Indicators for Sorbents of Air-Purifying Respirators.

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EARL W. MORGAN

Staff Scientist, Inhalation Toxicology Technical Group, Toxicology Department, Battelle, Pacific Northwest Laboratories

Educational Institutions Attended:

Civilian:

New Mexico State University	Physics: 1969-1971
University of Missouri-Columbia	BS, Animal Husbandry: 1971-1973
University of Missouri-Columbia College of Veterinary Medicine	DVM, Veterinary Medicine: 1972-1976
University of Kansas Medical	PhD Candidate (Toxicology): 1987

Military:

School of Aerospace Medicine Brooks AFB, TX	Veterinary Officer Basic: 1976
Academy of Health Sciences Fort Sam Houston, TX (correspondence): 1983-1984	Army Medical Department Officers Advanced Course
6227 US Army Reserve School Presidio of San Francisco, CA	Command and General Staff College (Seminar & Residence): 1984-1987

Research Background:

Four years applied toxicology (GLP).

Three years basic toxicology (Dissertation research on going).

One year aerosol toxicology including approximately 4 months post-doctoral training.

Administrative Background:

Supervised 4-14 veterinary technicians (Air Force and Marine).

Supervised Mammalian Section, Toxicology Branch, Letterman Army Institute Research. Section included: 2 DVM's, 1 PhD, and 5 BS level investigators and 6 animal caretakers.

Owned and operated a private veterinary practice with two employees.

Dr. Morgan served as chief of the Aerosol Biology Department. Supervised 3 PhD investigators and 3 technicians. Dr. Morgan was responsible for both basic and applied research on effects, mechanism of action, and prevention or prophylaxis of toxins of biological origins through aerosol exposure. He provided

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consulting and aerosol exposure support to other investigators within the Institute and U.S. Army Medical Research and Development Command.

Dr. Morgan performed an abbreviated post-doctoral training program in aerosol biology at the Battelle Pacific Northwest Laboratories; Richland, WA (Sep-Nov 90) and University of Rochester, NY (Nov-Dec 90) under the direction of Dr. Bernard Greenspan and Dr. Gunter Oberdorster, respectively prior to assuming my duties at USAMRIID.

Description of Previous Duties:

REDACTED

PhD Candidate
Kansas University Medical Center
Department of Pharmacology, Toxicology, and Therapeutics
Kansas City, KS

Position Description: Graduate student in toxicology at KUMC, see description of dissertation project attached to end. Plan to defend dissertation end of current semester.

REDACTED

Chief Mammalian Testing Section
Division of Toxicology
Letterman Army Institute Research
Presidio of San Francisco, CA

Position Description: Served as Mammalian Testing Section Chief. Supervised 7 professional investigators and 6 animal caretakers. Served as investigator for toxicological studies conducted in compliance with the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) Good Laboratory Practice Regulations. Prepared protocols, Standard Operating Procedures (SOP), and reports in compliance with Good Laboratory Practice Regulations. Conducted classical acute, subchronic, and chronic applied toxicological studies to include: LD_{50}, dermal and ocular irritation, dermal sensitivity, reproductive and teratology assays, 14, 28, and 180 day feeding studies, and a 2 year carcinogenicity bioassay as well as assisted with some Ames assays. Member of LAIR Animal Use Committee. Veterinarian for Toxicology Group animals.

REDACTED

Principal Investigator
Toxicology Section
Division of Comparative Medicine
Letterman Army Institute Research
Presidio of San Francisco, CA

Duties: Same as above except supervisory duties.

REDACTED

Private Veterinary Practice
Richland, MO and Oklahoma City, OK

Duties: Clinical Medicine. The practice at Richland, MO was a small town general veterinarian practice. However, it was predominately large animal, beef cow/calf and dairy. The practice at Oklahoma City was a 24 hour emergency hospital. The practice was predominately companion animals. However, we also

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treated a number of exotic pets as well as some large animals. Approximately 20% of the practice was equine medicine as the practice was co-owned and located with the Oklahoma Horse Shoeing School.

Base Veterinarian
Columbus AFB, MS REDACTED

Duties: Clinical Medicine, Preventative Medicine and Food Hygiene.

Chief Veterinary Service
Camp Pendleton MCB, CA REDACTED

Duties: Clinical Medicine, Preventative Medicine and Food Hygiene for three major Marine Corps Bases in southern California.

Base Veterinarian
Kunsan AFB, Korea REDACTED

Duties: Clinical Medicine, Preventative Medicine and Food Hygiene for southwest Korea; area included two Air Force Bases and four Army sites.

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Honors and Fellowships Received:

National Science Foundation Scholar in Physics, Asbury College - 1967

Deans List - New Mexico State University and University of Missouri-Columbia

American Animal Science Society Scholarship Award - 1971

Air Force Health Professions Scholarship Program

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Distinguished Graduate, Veterinary Officer Basic

AVMA Outstanding Student Award, Veterinary Officer Basic

Honor Graduate, Command and General Staff School

Expert Marksman

Air Force Commendation Medal

Army Commendation Medal

Special Appointments:

Hazardous Waste Disposal Officer while assigned at LAIR.

References:

Dr. Andrew Parkinson
Department of Pharmacology, Toxicology and Therapeutics
Kansas University Medical Center
Kansas City, Kansas 66103

Col. David R. Franz
Chief, Pathophysiology Division
U.S. Army Institute of Infectious Diseases
Ft. Detrick, Maryland 21702-5011

Dr. Richard C. Dorn
College of Veterinary Medicine
Ohio State University
Columbus, Ohio

Publications:

Papers:

Morgan E.W., Ho B., Brown L.D., Lewis C.M., Tillotson J.A., Lollini L.O., and Korte D.W. Jr.: Metabolism and Subchronic Toxicity of Nitroguanidine in the Rat. In: Proceedings of the Safety and Environmental Protection Subcommittee of the Joint Army-Navy-NASA-Air Force Interagency Propulsion Committee. Laurel, MD: Chemical Propulsion Information Agency, 1985. Note paper presented Nov 1985.

Hiatt G. F. S., Morgan E. W., Brown L. D., Lewis C. M., Johnson Y. C., Mullen L., Bauserman J. W., Okerburg C. V., Lollini L.O., and Korte D. W. Jr.: Acute Toxicity of Guanidine Nitrate and Nitroguanidine. In: Proceedings of the Safety and Environmental Protection Subcommittee of the Joint Army-Navy-NASA-Air Force Interagency Propulsion Committee. Laurel, MD: Chemical Propulsion Information Agency, 1985. Note paper presented Nov 1985.

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Brown L.D., Hiatt G.F.S., Morgan E.W., Wheeler C.R., Lewis C.M., Johnson Y.C., Ryabik J.R.G., Okerburg C.V., Makovec G.T., Lollini L.O., Mellick P.W., and Korte D.W. Jr.: Acute Toxicity of TEGDN and TMETN Liquid Propellents. In: Proceedings of the Safety and Environmental Protection Subcommittee of the Joint Army-Navy-NASA-Air Force Interagency Propulsion Committee. Laurel, MD: Chemical Propulsion Information Agency, 1985. Note paper presented Nov 1985.

Morgan E.W., Greenway D., and Parkinson A.: Purification of Two Rat liver Microsomal Carboxylesterases (Hydrolase A and B) With High Activity Towards *Para*-nitrophenylacetate. Archives of Biochemistry and Biophysics (paper submitted).

Morgan E.W., Greenway D., Wood G., and Parkinson A.: Regulation of Two Rat liver Microsomal Carboxylesterases (Hydrolase A and B). Archives of Biochemistry and Biophysics (paper submitted).

Morgan E.W. and Parkinson A.: Interindividual Variation and Tissue Distribution of Microsomal Carboxylesterase Activity in Humans. (In preparation).

Technical Reports:

1. Morgan E.W., Sano S.K., and Korte D.W. Jr.: Acute Oral Toxicity (LD_{50}) of Guanidine Hydrochloride in Male and Female Rats. Letterman Army Institute of Research (LAIR), Presidio of San Francisco, California Report No.204.

2. Mullen L., Morgan E.W., Lewis C.M., Wheeler C.R., and Korte D.W. Jr.: Acute Oral Toxicity of Guanidine Nitrate in Male and Female Rats. LAIR Report No. 267.

3. Hiatt G.F.S., Morgan E.W., and Korte D.W. Jr.: Dermal Sensitization of Guanidine Hydrochloride in Guinea Pigs. LAIR Report No. 210.

4. Morgan E.W., Bauserman J.W., and Korte D.W. Jr.: Primary Eye Irritation of Guanidine Nitrate in Male Rabbits. LAIR Report No. 212.

5. Morgan E.W., Hiatt G.F.S., and Korte D.W. Jr.: Acute Oral Toxicity (LD_{50}) of Guanidine Hydrochloride in Male and Female Mice. LAIR Report No 418.

6. Morgan E.W., Hiatt G.F.S., Lewis C.M., and Korte D.W. Jr.: Acute Oral Toxicity (LD_{50}) of (E)-1,2,3,4-Tetrahydro-6-Methyl-1-2-(2-Methyl-1-Oxo-2-Butenyl) Quinoline (CHR5) in Male and Female Rats. LAIR Report No. 217.

7. Morgan E.W., Mullen L, and Korte D.W. Jr.: Primary Dermal Irritation Potential of Guanidine Hydrochloride in Male and Female Rabbits. LAIR Report No. 213.

8. Morgan E.W., Sano S.K., and Korte D.W. Jr.: Primary Dermal Irritation Potential of Nitroguanidine in Male and Female Rabbits. LAIR Report.

9. Hiatt G.F.S., Morgan E.W., and Korte D.W. Jr.: Dermal Sensitization Potential of Guanidine Nitrate in Guinea Pigs. LAIR Report No. 282.

10. Sano S.K., Morgan E.W., and Korte D.W. Jr.: Mutagenic Potential of Hydroxylamine Hydrochloride (TP046). LAIR Report.

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11. Sano S.K., Morgan E.W., and Korte D.W. Jr.: Mutagenic Potential of Ballpowder®. LAIR Report No. 297.

12. Sano S.K., Morgan E.W., and Korte D.W. Jr.: Mutagenic Potential of Nitroguanidine (TP036). LAIR Report.

13. Morgan E.W., and Korte D.W. Jr.: Primary dermal Irritation Potential of Trimethylolethane Trinitrate (TMETN) in Rabbits. LAIR Report.

14. Morgan E.W., Hiatt G.F.S., and Korte D.W. Jr.: Dermal Sensitization Potential of Nitroguanidine in Guinea Pigs. LAIR Report No. 263.

15. Morgan E.W. and Korte D.W. Jr.: Acute Oral Toxicity of Ballpowder® in Sprague Dawley Rats. LAIR Report No 350.

16. Morgan E.W., Hiatt G.F.S., and Korte D.W. Jr.: Primary Eye Irritation Potential of Ballpowder® in Male Rabbits. LAIR Report No. 352.

17. Morgan E.W., Ryabik J.R.G., and Korte D.W. Jr.: Dermal Sensitization of Ballpowder® (Olin WC 844). LAIR Report.

18. Morgan E.W., Ryabik J.R.G., and Korte D.W. Jr.: Acute Oral Toxicity (LD_{50}) of Triethyleneglycol Dinitrate (TEGDN). LAIR Report.

19. Morgan E.W., Brown L.D., Lewis C.M., Dahlgren R.R., and Korte D.W. Jr.: Fourteen-Day Subchronic Oral Toxicity Study of Nitroguanidine in Rats. LAIR Report No. 272.

20. Morgan E.W Hiatt G.F.S., and Korte D.W. Jr.: Primary Dermal and Ocular Irritation Potential of 3-Chloro-4, 4-Dimethyl-2-Oxazolidione (Compound I). LAIR Report.

21. Morgan E.W. and Korte D.W. Jr.: Primary Dermal Irritation Potential of Nitrosoguanidine in Female Rabbits. LAIR Report No. 356.

22. Morgan E.W., Justus J.D., and Korte D.W. Jr.: Acute Dermal Toxicity of Nitrosoguanidine in Rabbits. LAIR Report No. 355.

23. Morgan E.W., Justus, J.D., and Korte D.W. Jr.: Primary Dermal Irritation Potential of JA_2 Solid Propellant in Rabbits. LAIR Report No. 413.

24. Morgan E.W. and Korte D.W. Jr.: Acute Dermal Toxicity (MLD) of Triethyleneglycol Dinitrate (TEGDN). LAIR Report No. 342.

25. Morgan E.W. and Korte D.W. Jr.: Acute Dermal Toxicity of Trimethylolethane Trinitrate (TMETN) in Rabbits. LAIR Report No 347.

26. Morgan E.W., Frost D.F. Wheeler C.R., and Korte D.W. Jr.: Acute Oral Toxicity of JA_2 Solid Propellant in ICR Mice. LAIR Report No. 414.

27. Morgan E.W., Brogden J.D., and Korte D.W. Jr.: Primary Eye Irritation Potential of JA_2 Solid Propellant in Rabbits. LAIR Report No. 415.

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28. Morgan E.W. and Korte D.W. Jr.: Acute Oral Toxicity of Nitrosoguanidine in Sprague Dawley Rats. LAIR Report No. 354.

29. Morgan E.W., Wheeler C.R. and Korte D.W. Jr.: Acute Oral Toxicity of Nitrosoguanidine in ICR Mice. LAIR Report No. 354.

30. Morgan E.W., Justus J.D., Frost D.F., and Korte D.W. Jr.: Dermal Sensitization Potential of Nitrosoguanidine in Guinea Pigs. LAIR Report No. 357.

31. Morgan E.W., Pearce M.J., Zaucha G.M., Lewis C.M., Makovec G.T., and Korte D.W. Jr.: Ninety-Day Subchronic Oral Toxicity Study of Nitroguanidine in Rats. LAIR Report No. 306.

32. Magnuson D.K., Morgan E.W., and Korte D.W. Jr.: Primary Dermal Irritation Potential of Physostigmine Salicylate. LAIR Report No. 331.

33. Frost D.F., Morgan E.W., Letellier Y.C., Pearce M.J., Ferraris S, Smith C.D., Zaucha G.M., and Korte D.W. Jr.: Ninety-Day Subchronic Oral Toxicity Study of Nitroguanidine in Mice. LAIR Report No. 319.

34. Morgan E.W., Zaucha G.M., Waring P.P., Letellier Y.C., Seewald J.B., Clifford C.B., and Korte D.W. Jr.: Ninety-Day Subchronic Oral Toxicity Study of Pyridostigmine Bromide in Rats. LAIR Report No. 435.

35. Morgan E.W., Zaucha G.M., Waring P.P., Letellier Y.C., Seewald J.B., Clifford C.B., and Korte D.W. Jr.: One Hundred Eighty Day Subchronic Oral Toxicity Study of Pyridostigmine Bromide in Rats. LAIR Report No. 441.

36. Morgan E.W., Zaucha G.M., Bowman P., Schuschereba S.T., Waring P.P., Letellier Y.C., Seewald J.B., and Korte D.W. Jr.: Ninety-Day Subchronic Oral Toxicity Study of Pyridostigmine Bromide in Rats - Electron Microscopy Study of Neuromuscular Junctions. LAIR Report No. 436.

Abstracts:

1. Morgan E.W., Wood G., Greenway D.J., and Parkinson A.: Developmental Changes in the Levels of a Rat Liver Microsomal Carboxyesterase (Hydrolase A). The Toxicologist 1989;9:39. Note paper presented 28 Feb 89.

2. Lewis C.M., Morgan E.W., Brown L.D., Pearce M.J., Zaucha G.M., Makovec G.T., and Korte D.W. Jr.: 90-Day Oral Subchronic Toxicity of Nitroguanidine in Rats. The Toxicologist 1989;9:425. Note poster presented 28 Feb 89.

3. Morgan E.W. and Parkinson A.: Comparison of the tissue distribution of two isozymes of rat liver microsomal carboxyesterase (hydrolases A and B). The Toxicologist 1990;10:127. Note paper presented 14 Feb 90.

4. Yan B, Morgan E.W., and Parkinson A.: Immunocytochemical localization of two carboxyesterases (hydrolases A and B) in rat liver, testis, and kidney. The Toxicologist 1990;10:323. Note poster presented 16 Feb 90.

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5. Parkinson A., Morgan E.W., and Yan B.: Purification and characterization of two rat liver microsomal carboxyesterases; studies on their regulation and tissue distribution In: Proceedings of the Eighth International Symposium on Microsomes and Drug Oxidations. Karolinska Institute, Stockholm, Sweden, 1990. Note presented Jun 90.

6. Yan B, Morgan E.W., Parkinson A.: Esterase activity in human liver, kidney, and testis. The Toxicologist 1991;11:228. Note poster presented on 28 Feb 90.

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NAZLI EGILMEZ-REYNOLDS

Senior Research Scientist, Aerosol and Inhalation Research, Toxicology Department, Battelle, Pacific Northwest Laboratories

Ph.D Chemistry (1980-1983), School of Science and Engineering, University of Essex, England.
M.Sc Chemistry (1978-1980), School of Science and Engineering, University of Essex, England.
B.Sc Chemical Engineering (1974-1978), Aegean University, Turkey.

CAREER HISTORY AND ACCOMPLISHMENTS

*University of Louisville; Chemistry Department (August 1988 - May 1991)

Visiting Assistant Professor

Full-time instructor of General Chemistry (100 and 200 level) lectures and laboratories.

- Taught introductory chemistry course to a mixture of students (majors and non-majors) for seven consecutive semesters, totalling some 1250 students in thirteen sections. Used instructional aids frequently.
- Organized and supervised 100 and 200 level laboratory courses on General Chemistry as the Senior Instructor. Supervised a total of 16 teaching assistants from six different countries.
- Taught introductory Analytical Chemistry course with lecture and laboratory.
- Formed close contacts with the students, providing necessary counseling and additional help in the form of study sessions outside office hours (see the letter from the Board of Trustees and the student evaluations enclosed).
- Prepared a graduate level course on Aerosol Science and Technology upon an invitation by the Chemical Engineering Department of Speed School of U of L.
- *Self-employed Aerosol Scientist (July 1987 - Present)

Scientific Consultant on Aerosol Science and Technology/Tobacco Smoke

Advisor on the formation, measurement, filtration and behavior of Aerosol Systems and their interaction with humans and the environment. The following companies have been clients:

- British-American Tobacco Co., Hamburg, Germany (BAT Cigaretten Fabriken Gmb)
- Brown and Williamson Tobacco Co., Louisville, USA.
- C.A. Blockers, Inc., Louisville, USA.

*British-American Tobacco UK & Export Ltd., Southampton, England (1983-1987)

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Research Scientist - Product Research Section

Responsible for the Aerosol Laboratory; Chief Aerosol Scientist for the BAT Group worldwide.

- Developed and executed work programs on aerosol generation and behavior inside the cigarette, filters, human upper airways and environmental rooms.
- Provided an expert advisory service to complement the needs of other BAT Research Centers and R&D Sections.
- Coordinated research with associated laboratories in Louisville (USA), Hamburg (Germany) and Montreal (Canada).
- Identified and devised instrumentation and data-logging facilities; organized, conducted and interpreted experimental studies and communicated the results and implications throughout BAT by writing reports and giving verbal presentations.
- Developed synthetic smoke generation techniques and used model aerosols to investigate the relation between smoke taste and its aerosol characteristics.
- Produced guidelines and provided advice on the design of new products. Worked on the concept of alternative products and designed two hand-held delivery systems.
- Used specialized aerosol equipment by TSI, PMS, and ROYCO, interfaced with Apple and IBM PCs, in the generation and counting of particles.

*University of Essex, Colchester, England (1980-1983)

Research Assistant, Department of Chemistry

Reported to the Director of the Aerosol Lab., Prof. C.N. Davies.

- Developed and characterized a synthetic aerosol generator of $CaCO_3$ in nanometer particle radius.
- Performed coagulation experiments using monodisperse and polydisperse aerosols at varying particle sizes and concentrations, demonstrating the large discrepancy between theory and experiments.
- Developed a new theory of aerosol coagulation for submicron aerosol particles in the light of experimental results.
- Evaluated the losses in a Nolan-Pollack Counter and demonstrated the validity of its calibration.
- Reviewed papers as a referee for the Journal of Aerosol Science.

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R: REDACTED MATERIAL

AWARDS

- Research Assistantship (1980-1983) offered by the SERC of Britain
- British Council Award (1979-1980)
- Fulbright Scholarship (1978)

REDACTED

REDACTED

PUBLICATIONS

Egilmez, N. and Davies, C.N. (1986) "Aerosols: Formation and Reactivity", Chapter 6, Pergamon Press, London, 453.

Davies, C.N. and Egilmez, N. (1986) "Nolan-Pollack Condensation Nucleus Counter", Letter to the Editor, Aerosol Science and Technology, 5, 117.

Davies, C.N. and Egilmez, N. (1984) "An Investigation into the Loss of Particles by Diffusion in the Nolan-Pollack CNC", Journal of Aerosol Science, 15, 177-181.

Egilmez, N. and Davies, C.N. (1982) "Coagulation of Fine Aerosol", Proceedings of Royal Society of London, A 380, 99.

Egilmez, N. and Davies, C.N. (1981) "Coagulation of Fine Aerosols", Journal of Aerosol Science, 12, 184.

RAT REPORTS (1983-1987; restricted for open publication):

- Review of Studies on Cigarette Smoke Aerosol Formation
- Generation of Two-Component, Relatively Monodisperse Aerosols
- Evaluation of a Patented Noncombustible Cigarette
- Aerosol Particle Formation During the Heating of Tobacco
- Joint Aerosol Studies in Chemosensory Research
- Ambient Smoke Aerosol Characterization
- Smoke Aerosol Characterization of Some Slim and Low-tar Cigarettes from the U.S. Market
- Development and Calibration of a Synthetic Aerosol Generator

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- A Preliminary Study of Aerosol Characteristics Inside a Glass Model of the Human Upper Airways
- Aerosol Characterization of Smoke From Cigarettes Tipped With Extreme Design Filters
- Sensory Perception Assessment of Synthetic Aerosols

PRESENTATIONS

SECOND INTERNATIONAL AEROSOL CONFERENCE, West Berlin, Germany, September 22-26, 1986; "The Coagulation of Fine Aerosols".

EUROMEC COLLOQUIUM, University of Cambridge, Cambridge, England, April 1985; "Coagulation of Fine Aerosols Alone and When Mixed With a Coarse Aerosol".

EUROMEC COLLOQUIUM, University College, London, England, September 1982; "Coagulation of Aerosols--a Discussion of the Various Regimes of Coagulation and Their Experimental Basis".

INTERNATIONAL AEROSOL CONFERENCE, Schmallenberg, Germany, September 20-24, 1980; "Coagulation of Submicron Aerosols".

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R: REDACTED MATERIAL

JOHN Y. YOUNG

Health and Safety Officer, Inhalation Toxicology Technical Group, Toxicology Department, Battelle, Pacific Northwest Laboratories

Education:

University of Washington, Seattle, WA

1980 Master of Science in Public Health (Industrial Hygiene)
Thesis Title: Personnel Exposure to Methylmethacrylate in Operating Theaters
1971 Bachelor of Science (Preventive Medicine)

Certifications:

Diplomate, American Academy of Industrial Hygiene - Certified in the Comprehensive Practice of Industrial Hygiene
Diplomate, American Academy of Sanitarians
Registered Sanitarian - National Environmental Health Association
Registered Sanitarian - Board for Registration of Sanitarians in Pennsylvania

Summary of Experience:

Mr. Young has planned and implemented industrial hygiene and environmental protection program for conduct of animal studies involving toxic and potentially carcinogenic chemicals. He has twenty years of military service as a U.S. Army environmental science officer specializing in industrial hygiene, with experience in industrial hygiene research and consulting, rural sanitation projects and training, military installation environmental compliance, and health surveillance activities. Extensive experience in the past six years in conducting toxic hazard research and in administering an occupational health research program for the U.S. Army.

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U.S. Army Biomedical Research and Development Laboratory, Fort Detrick, MD

1990-1992: CHIEF, OCCUPATIONAL HEALTH RESEARCH BRANCH

- Managed a research branch of an Army research and development laboratory, responsible for planning research projects and programming resources and budget for both in-house and extramural occupational health research.

- Supervised 9 research scientists involved in medical research to study the health effects of military-unique chemical hazards, to conduct exposure assessment studies on soldiers' exposures to smoke and weapons combustion products, and to develop innovative techniques for military field water and sanitation.

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- Interacted extensively with Army medical units and major Army system and material developers, and served as a consultant to these organizations on industrial hygiene related matters, and

- Served as a Source Selection Board member involved in the evaluation and selection of contractors for Army system and material development programs.

1986-1990: ENVIRONMENTAL SCIENCE OFFICER/INDUSTRIAL HYGIENIST

- Managed and conducted both in-house and extramural occupational health research projects, served as a research team leader, and solicited/coordinated field support

  - to evaluate soldiers' exposure to:

    - weapons combustion products inside armored vehicles
    - deliberately-generated smoke in military field training
    - rocket and missile launch combustion products

  - to characterize:

    - physical/chemical compositions of propellant combustion products
    - particle size distribution of experimental obscuration smoke
    - dispersion modeling of obscuration smoke

  - to develop data base on:

    - medical criteria for military respiratory protection
    - training scenarios involving the use of obscuration smoke

- Provided consultation services to U.S. Army Missile Command on toxic hazards associated with major weapon design and procurement. This involved defining industrial hygiene air sampling procedures during prototype weapon testing, reviewing contractor's design proposals, and establishing acceptability criteria regarding occupational health protection issues.

- Maintained equipment calibration, planned equipment requirements for future industrial hygiene research, prepared various research protocols and developed quality control procedures to improve this laboratory's occupational health research program.

<u>7th Infantry Division (Light), Fort Ord, CA</u>

1984-1986: COMMANDER, 172D MEDICAL DETACHMENT

- Commanded a preventive medicine detachment of 11 personnel. Duties included administrative personnel actions, combat-readiness training, and professional development of assigned personnel.

- Supported the 7th Infantry Division regularly on field training maneuvers to monitor and improve sanitary conditions of field water supplies, food services, and bivouac areas. Also supported the 7th Infantry Division to process units being deployed overseas.

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- Provided a monthly 1-week training course on field sanitation to assigned units in the 7th Infantry Division, and to other active and reserve military units in the Monterey area.

U.S. Army Pacific Environmental Health Engineering Agency, U.S. Army in Japan

1980-1984: CHIEF, INDUSTRIAL HYGIENE DIVISION

- Performed administrative functions of a small industrial hygiene division and supervised 2 industrial hygienists and one industrial hygiene technician.
- Planned and programmed resources to implement an industrial hygiene surveillance/support program for Army installations in Japan, Korea, Kwajelein Islands, and Hawaii. Also served the Department of Defense, U.S. Air Forces, U.S. Navy, and U.S. Coast Guard as needed in Japan, Korea, Guam, Yap, and the Philippines.
- Conducted comprehensive industrial hygiene surveys and hearing conservation surveys on all supported installations by following a schedule of at least one visit every 2 years.
- Received requests and conducted industrial hygiene special studies on specific health hazards such as asbestos, benzene, mercury, lead, carbon monoxide, pentachlorophenol, polychlorinated biphenyls, hexachloroethane, and noise.
- Established a laboratory to identify bulk asbestos samples using the polarized light microscopy technique.

Letterman Army Medical Center, Presidio of San Francisco, CA

1974-1978: ENVIRONMENTAL SANITARIAN

- Supervised up to 18 environmental health technicians and reported to a preventive medicine physician.
- Provided the full spectrum of environmental health surveillance and support in the Army and Department of Defense facilities in Northern California. Support services included drinking water supply surveillance; air pollution source monitoring; sanitary inspection of food service/ice-making facilities; barber/beauty shops; child care facilities; detentional cells; solid waste disposal planning; hospital infection control; operating room clean air monitoring; routine industrial hygiene support; pesticide monitoring; and health status documentation and reporting.

1st Special Forces Group, U.S. Army in Okinawa

1973-1974: PUBLIC HEALTH TEAM LEADER, CIVIL AFFAIRS

- Planned special forces civil affairs sanitation projects.
- Visited remote villages in the Philippines to provide classroom instructions to local doctors, nurses, sanitarians, and school teachers on sanitation and disease prevention.

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- Constructed water catchment tanks to protect village water supplies.
- Constructed and installed water-sealed toilet facilities to provide a sanitary means for human fecal disposal.

Academy of Health Sciences, U.S. Army, Fort Sam Houston, TX

1972-1973: PREVENTIVE MEDICINE INSTRUCTOR

- Served as an instructor and advisor for students attending the Army Preventive Medicine Basic (91S10) Course.
- Prepared lesson plans for the 91S10 and the Advanced (91S20) courses.
- Provided classroom support when not involved in class instruction.

RECENT PUBLICATIONS:

Technical Reports:

Young, J.Y., D.A. Smart, J.T. Allen, D.L. Parmer, A.B. Rosencrance, E.E. Brueggemann, and F.H. Broski. September 1989. Field exposure of chemical school students and cadre to fog oil and hexachloroethane (HC) smokes. U.S. Army Biomedical Research and Development Laboratory Technical Report 8908. Defense Technical Information Center Publication No. AD A225008.

Eaton, J.E. and J.Y. Young. January 1989. Medical criteria for respiratory protection in smoke: the effectiveness of the military protective mask. U.S. Army Biomedical Research and Development Laboratory Technical Report 8902. Defense Technical Information Center Publication No. AD A204303.

Smart, D.A., D.L. Parmer, J.Y. Young, W.G. Palmer. In press. The relationship between doctrine for Army tactical smoke use and exposure potential: priorities for conducting smoke exposure assessment studies. U.S. Army Biomedical Research and Development Laboratory Technical Report 9115.

Proceedings:

Young, J.Y., D.A. Smart, D.L. Parmer, J.T. Allen, and S.H. Hoke. April 1988. Measurement of field exposure to fog oil and hexachloroethane smokes. In Proceedings of the smoke/obscurants symposium XII, ed. W.M. Farmer and W. Klimet. Aberdeen Proving Ground, MD: Office of the Program Manager for Smoke/Obscurants.

Young, J.Y. September 1988. Exposure assessment related to smoke/obscurants. In Proceedings of the tri-service pulmonary research review and analysis in Denver, Colorado. Fort Detrick, MD: U.S. Army Medical Research and Development Command.

Young, J.Y. September 1988. Evaluation of breathing-zone exposure to AAWS-M exhaust combustion products. In Proceedings of the tri-service pulmonary research review and analysis in Denver, Colorado. Fort Detrick, MD: U.S. Army Medical Research and Development Command.

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COST ELEMENT INFORMATION

DIRECT LABOR

Direct labor costs are based on average charge-out rates for specific job categories.

LABOR OVERHEAD

Building and Utilities
The estimated rates per direct labor hour for FY 1993 through FY 1997 are $6.85, $6.90, $7.40, $7.20, and $7.30, respectively. Building and Utilities are costs for occupying U.S. Department of Energy (DOE) or Battelle facilities. Typical costs include utilities, building operations, janitors, maintenance, insurance, and taxes. The rates have been approved in our Indirect Cost Rate Agreement with the Department of Energy.

Organizational Overhead
Organizational Overhead for technical organizations represents costs for management, supervision, and administration of technical departments. Organizational Overhead also includes costs for research equipment such as small tools, lab supplies, laundry, decontamination/waste disposal, maintenance, and expenses associated with Battelle owned equipment with an initial cost less than $50,000. Also included is a factor for reimbursement to the Government for use of DOE owned equipment with an initial cost less than $50,000 for work performed under Contract DE-AC06-76RLO-1831.

Organizational Overhead for non-technical organizations represents costs for department management and support. These costs are accumulated in an intermediate cost pool and are allocated to cost objectives at a predetermined rate per direct labor hour.

TRAVEL

Airfare rates have been estimated utilizing quotes from local travel agencies. Subsistence costs (meals and lodging) have been estimated using per diem rates published in the Federal Travel Regulations. Travel costs have been escalated at an annual rate of 5.0 percent.

OTHER DIRECT COSTS

BNW Procurement Services
Procurement Service Charge includes costs for procuring and receiving purchased items. For estimating purposes, an average service charge of 10% has been applied to materials (plus escalation).

Actual Procurement service charges are as follows:

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Purchase Order Amount	Charge
$100 and under	$50
Next $4,900	10%
All Over $5,000	5% (maximum of $5,290)

Subcontracts & Consulting Services
Subcontracts and Consulting service charge includes costs for placing and administering subcontracts and consultants. Subcontracts & Consulting service charges are estimated on the following basis: 8.0 percent of the total cost per subcontract up to a maximum of $25,000 per fiscal year.

Other Direct Costs
The cost of certain centralized services such as duplicating, technical information, computer usage, etc., are accumulated in an intermediate cost pool and are allocated based on the actual usage of the services provided by each center.

Special Purpose Equipment

Battelle-Owned Equipment
Costs associated with major items/systems of Battelle-owned equipment (in excess of $50,000 initial cost) are accumulated in an intermediate cost pool and allocated at a predetermined usage rate for each item.

Government-Owned Equipment
The estimated cost of Government-owned equipment usage is based on reimbursement rates established by contractual agreement between BNW and the Department of Energy. (Contract No. DE-AC06-76RLO-1831)

Cost of Facilities Capital (CAS 414)
The current estimated rates are $.87 per direct labor hour for personnel located in Battelle buildings, and $.30 per direct labor hour for personnel located in Department of Energy (DOE) buildings. The rates have been approved in our Indirect Cost Rate Agreement with the Department of Energy.

STORES OVERHEAD
The estimated cost for stores overhead is based upon a Westinghouse Hanford Company rate of 15.2 percent of store items.

GENERAL AND ADMINISTRATIVE EXPENSE
G&A includes general functions such as Accounting, Legal, and Personnel department costs, and costs for bid and proposal, selling, IR&D, contract administration, etc. G&A is allocated at the estimated rate of 45.0 percent of value added to final cost objectives.

ANIMAL RESOURCE CENTER
The Animal Resource Center (ARC) is a service center within the Toxicology Department. The ARC is responsible for routine animal

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care, animal health and management of experimental animals. The costs associated with this activity are accumulated in an intermediate cost pool and allocated to cost objectives at predetermined rates by the type of housing provided.

These rates incorporate costs for basic animal care, including costs associated with basic animal health, food, materials and equipment depreciation and maintenance cost. Basic animal care consists of the labor for all routine animal care: feeding and watering the animals; cleaning the rooms housing the animals, hallways, and cages; and supervision of the technicians and management of the Animal Resource Center. Basic health care of the animals is defined as routine monitoring of sanitation, water quality, feed and bedding, clinical observation for infectious disease and the initial health screening. Costs associated with activities above the basic level of animal care service (such as non-stock cage board), are treated as direct costs.

OVERTIME POLICY

The responsibilities of exempt staff members cannot always be met within regular work schedules, and extra time and effort is taken into consideration in determining base salary ranges for all positions as well as in the extra vacation allowance for exempt staff. However, when scheduled work time requirements beyond 40 hours per week are regular, consistent, and clearly in excess of that reasonably expected for the position, overtime payments for exempt staff members will be considered. Nonexempt staff members will receive overtime and premium pay for their overtime work.

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COST SUMMARY
BASE STUDY
20643

	TOTAL	PHASE 1	CHRONIC YEARS
1. DIRECT LABOR			
RESEARCH SCIENTISTS/ENGINEERS	$1,764,977	$317,494	$1,447,483
TECHNICAL SPECIALIST	$1,461,209	$187,714	$1,273,495
ADMINISTRATIVE PROF.	$39,289	$2,577	$36,712
TECHNICIANS	$707,947	$66,036	$641,911
CRAFTS	$127,272	$86,203	$41,069
CLERICAL/SECRETARIAL	$90,335	$12,426	$77,909
TOTAL DIRECT LABOR	$4,191,029	$672,450	$3,518,579
2. LABOR OVERHEAD			
BUILDING AND UTILITIES	$720,517	$140,294	$580,223
ORGANIZATIONAL OVERHEAD	$2,258,722	$371,677	$1,887,045
TOTAL LABOR OVERHEAD	$2,979,239	$511,971	$2,467,268
3. TRAVEL			
TRANSPORTATION	$14,959	$4,385	$10,574
SUBSISTENCE	$3,841	$1,126	$2,715
TOTAL TRAVEL	$18,800	$5,511	$13,289
4. OTHER DIRECT COSTS			
NON LABOR DIRECT COSTS	$2,649,093	$254,083	$2,395,010
5. VALUE ADDED	$9,838,161	$1,444,015	$8,394,146
6. OTHER INDIRECT COSTS			
CAS 414	$82,682	$15,104	$67,578
TOTAL OTHER INDIR. COST	$82,682	$15,104	$67,578
7. DIRECT MATERIALS			
MISCELLANEOUS MATERIALS	$606,229	$157,820	$448,409
CAPITAL EQUIPMENT	$539,969	$539,969	$0
SUBCONT./CONSULTING & OHC	$47,300	$5,700	$41,600
TOTAL DIRECT MATERIALS	$1,193,498	$703,489	$490,009
8. GENERAL & ADMIN. EXPENSE	$4,427,174	$649,807	$3,777,367
9. SUBTOTAL	$15,541,515	$2,812,415	$12,729,100
10. FEE	$1,545,886	$279,731	$1,266,155
11. TOTAL COST	$17,087,401	$3,092,146	$13,995,255

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COST SUMMARY
CHRONIC YEARS
20643

Base Contract

	TOTAL	CHRONIC YEAR 1		EAR 3	CHRONIC YEAR 4
1. DIRECT LABOR					
RESEARCH SCIENTISTS/ENGINEERS	$1,447,483	$497,079		43	$93,837
TECHNICAL SPECIALIST	$1,273,495	$454,717	$475,924	$331,331	$11,523
ADMINISTRATIVE PROF.	$36,712	$9,275	$10,687	$11,728	$5,022
TECHNICIANS	$641,911	$287,054	$306,827	$41,213	$4,817
CRAFTS	$41,069	$14,902	$19,167	$6,947	$53
CLERICAL/SECRETARIAL	$77,909	$22,628	$26,268	$19,275	$9,738
TOTAL DIRECT LABOR	$3,518,579	$1,285,655	$1,349,697	$758,237	$124,990
2. LABOR OVERHEAD					
BUILDING AND UTILITIES	$580,223	$234,805	$227,496	$104,264	$13,658
ORGANIZATIONAL OVERHEAD	$1,887,045	$735,545	$735,329	$349,958	$66,213
TOTAL LABOR OVERHEAD	$2,467,268	$970,350	$962,825	$454,222	$79,871
3. TRAVEL					
TRANSPORTATION	$10,574	$0	$5,183	$5,391	$0
SUBSISTENCE	$2,715	$0	$1,331	$1,384	$0
TOTAL TRAVEL	$13,289	$0	$6,514	$6,775	$0
4. OTHER DIRECT COSTS					
NON LABOR DIRECT COSTS	$2,395,010	$1,175,030	$1,146,495	$73,321	$164
5. VALUE ADDED	$8,394,146	$3,431,035	$3,465,531	$1,292,555	$205,025
6. OTHER INDIRECT COSTS					
CAS 414)	$67,578	$27,365	$26,161	$11,884	$2,168
TOTAL OTHER INDIR. COST	$67,578	$27,365	$26,161	$11,884	$2,168
7. DIRECT MATERIALS					
MISCELLANEOUS MATERIALS	$448,409	$253,942	$186,270	$7,204	$993
SUBCONT./CONSULTING & OHC	$41,600	$17,600	$16,500	$7,500	$0
TOTAL DIRECT MATERIALS	$490,009	$271,542	$202,770	$14,704	$993
8. GENERAL & ADMIN. EXPENSE	$3,777,367	$1,543,965	$1,559,490	$581,651	$92,261
9. SUBTOTAL	$12,729,100	$5,273,907	$5,253,952	$1,900,794	$300,447
10. FEE	$1,266,155	$524,655	$522,780	$188,892	$29,828
11. TOTAL COST	$13,995,255	$5,798,562	$5,776,732	$2,089,686	$330,275

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Base Control

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COST SUMMARY
CHRONIC YEARS
20643

	TOTAL	CHRONIC YEAR 1	CHRONIC YEAR 2	CHRONIC YEAR 3	CHRONIC YEAR 4
1. DIRECT LABOR					
RESEARCH SCIENTISTS/ENGINEERS	$1,447,483	$497,079	$506,824	$347,743	$93,837
TECHNICAL SPECIALIST	$1,273,495	$454,717	$475,924	$331,331	$11,523
ADMINISTRATIVE PROF.	$36,712	$9,275	$10,687	$11,728	$5,022
TECHNICIANS	$641,911	$287,054	$308,827	$41,213	$4,817
CRAFTS	$41,069	$14,902	$19,167	$6,947	$53
CLERICAL/SECRETARIAL	$77,909	$22,628	$26,268	$19,275	$9,738
TOTAL DIRECT LABOR	$3,518,579	$1,285,655	$1,349,697	$758,237	$124,990
2. LABOR OVERHEAD					
BUILDING AND UTILITIES	$580,223	$234,805	$227,496	$104,264	$13,658
ORGANIZATIONAL OVERHEAD	$1,887,045	$735,545	$735,329	$349,958	$66,213
TOTAL LABOR OVERHEAD	$2,467,268	$970,350	$962,825	$454,222	$79,871
3. TRAVEL					
TRANSPORTATION	$10,574	$0	$5,183	$5,391	$0
SUBSISTENCE	$2,715	$0	$1,331	$1,384	$0
TOTAL TRAVEL	$13,289	$0	$6,514	$6,775	$0
4. OTHER DIRECT COSTS					
NON LABOR DIRECT COSTS	$2,395,010	$1,175,030	$1,146,495	$73,321	$164
5. VALUE ADDED	$8,394,146	$3,431,035	$3,465,531	$1,292,555	$205,025
6. OTHER INDIRECT COSTS					
CAS 414)	$67,578	$27,365	$26,161	$11,884	$2,168
TOTAL OTHER INDIR. COST	$67,578	$27,365	$26,161	$11,884	$2,168
7. DIRECT MATERIALS					
MISCELLANEOUS MATERIALS	$448,409	$253,942	$186,270	$7,204	$993
SUBCONT./CONSULTING & OHC	$41,600	$17,600	$16,500	$7,500	$0
TOTAL DIRECT MATERIALS	$490,009	$271,542	$202,770	$14,704	$993
8. GENERAL & ADMIN. EXPENSE	$3,777,367	$1,543,965	$1,559,490	$581,651	$92,261
9. SUBTOTAL	$12,729,100	$5,273,907	$5,253,952	$1,900,794	$300,447
10. FEE	$1,266,155	$524,655	$522,780	$188,892	$29,828
11. TOTAL COST	$13,995,255	$5,798,562	$5,776,732	$2,089,686	$330,275

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COST SUMMARY
OPTIONS
20643

	SUBCHRONIC	P-450/GST ASSAYS	BIG BLUE MOUSE	PROTEIN ADDUCTS	MICRONUCLEUS ASSAY	PCNA
1. DIRECT LABOR						
RESEARCH SCIENTISTS/ENGINEERS	$267,628	$24,648	$13,376	$45,012	$15,824	$10,362
TECHNICAL SPECIALIST	$148,321	$22,818	$12,578	$25,203	$10,664	$9,145
ADMINISTRATIVE PROF.	$7,708	$0	$0	$0	$0	$0
TECHNICIANS	$101,161	$0	$4,168	$0	$2,194	$3,093
CRAFTS	$5,463	$0	$0	$0	$0	$0
CLERICAL/SECRETARIAL	$21,548	$0	$0	$0	$0	$0
TOTAL DIRECT LABOR	$551,829	$47,466	[illegible]			$22,600
2. LABOR OVERHEAD						
BUILDING AND UTILITIES	$106,012					$3,785
ORGANIZATIONAL OVERHEAD	$312,749					$11,118
TOTAL LABOR OVERHEAD	$418,761					$14,903
3. TRAVEL						
TRANSPORTATION	$4,710					$0
SUBSISTENCE	$1,209					$0
TOTAL TRAVEL	$5,919					$0
4. OTHER DIRECT COSTS						
NON LABOR DIRECT COSTS	$304,160					$264
5. VALUE ADDED	$1,280,669					$37,767
6. OTHER INDIRECT COSTS						
CAS 414	$12,594	$754	$395	$1,279	$598	$455
TOTAL OTHER INDIR. COST	$12,594	$754	$395	$1,279	$598	$455
7. DIRECT MATERIALS						
MISCELLANEOUS MATERIALS	$80,856	$36,650	$44,874	$32,869	$4,401	$2,826
SUBCONT./CONSULTING & OHC	$11,200	$0	$0	$0	$0	$0
TOTAL DIRECT MATERIALS	$92,056	$36,650	$44,874	$32,869	$4,401	$2,826
8. GENERAL & ADMIN. EXPENSE	$576,301	$37,871	$28,447	$65,682	$23,439	$16,995
9. SUBTOTAL	$1,961,620	$161,432	$136,931	$245,789	$80,524	$58,043
10. FEE	$194,904	$16,068	$13,654	$24,451	$7,993	$5,759
11. TOTAL COST	$2,156,524	$177,500	$150,585	$270,240	$88,517	$63,802

Subchronic doesn't include set up or purchase of exposure generation equip.

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Subchronic
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generation equip.

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COST SUMMARY
OPTIONS
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	SUBCHRONIC	P-450/GST ASSAYS	BIG BLUE MOUSE	PROTEIN ADDUCTS	MICRONUCLEUS ASSAY	PCNA
1. DIRECT LABOR						
RESEARCH SCIENTISTS/ENGINEERS	$267,628	$24,648	$13,376	$45,012	$15,824	$10,362
TECHNICAL SPECIALIST	$148,321	$22,818	$12,578	$25,203	$10,664	$9,145
ADMINISTRATIVE PROF.	$7,708	$0	$0	$0	$0	$0
TECHNICIANS	$101,161	$0	$4,168	$0	$2,194	$3,093
CRAFTS	$5,463	$0	$0	$0	$0	$0
CLERICAL/SECRETARIAL	$21,548	$0	$0	$0	$0	$0
TOTAL DIRECT LABOR	$551,829	$47,466	$30,122	$70,215	$28,682	$22,600
2. LABOR OVERHEAD						
BUILDING AND UTILITIES	$106,012	$7,671	$6,079	$12,157	$5,355	$3,785
ORGANIZATIONAL OVERHEAD	$312,749	$24,932	$22,829	$38,947	$16,360	$11,118
TOTAL LABOR OVERHEAD	$418,761	$32,603	$28,908	$51,104	$21,715	$14,903
3. TRAVEL						
TRANSPORTATION	$4,710	$0	$0	$0	$0	$0
SUBSISTENCE	$1,209	$0	$0	$0	$0	$0
TOTAL TRAVEL	$5,919	$0	$0	$0	$0	$0
4. OTHER DIRECT COSTS						
NON LABOR DIRECT COSTS	$304,160	$4,088	$4,185	$24,640	$1,689	$264
5. VALUE ADDED	$1,280,669	$84,157	$63,215	$145,959	$52,086	$37,767
6. OTHER INDIRECT COSTS						
CAS 414	$12,594	$754	$395	$1,279	$598	$455
TOTAL OTHER INDIR. COST	$12,594	$754	$395	$1,279	$598	$455
7. DIRECT MATERIALS						
MISCELLANEOUS MATERIALS	$80,856	$38,650	$44,874	$32,869	$4,401	$2,826
SUBCONT./CONSULTING & OHC	$11,200	$0	$0	$0	$0	$0
TOTAL DIRECT MATERIALS	$92,056	$38,650	$44,874	$32,869	$4,401	$2,826
8. GENERAL & ADMIN. EXPENSE	$576,301	$37,871	$28,447	$65,682	$23,439	$16,995
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11. TOTAL COST	$2,156,524	$177,500	$150,585	$270,240	$88,517	$63,802

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PROVISIONS FOR CONTRACTS UTILIZING GOVERNMENT-OWNED FACILITIES AND EQUIPMENT, OR CERTAIN UNREPORTED U. S. DEPARTMENT OF ENERGY INFORMATION

It is planned that the work, or a portion thereof, to be performed hereunder will be performed at the Pacific Northwest Laboratory (hereinafter called the Laboratory), owned by the U. S. Department of Energy (hereinafter called DOE) operated for DOE by BATTELLE under Operating Contract DE-AC06-76RLO 1830 (hereinafter called Operating Contract), and used by BATTELLE for its own account under and subject to the Use Permit Contract DE-AC06-76RLO 1831 (hereinafter called Use Permit). Therefore, performance of the work hereunder shall be contingent upon the continuing availability to BATTELLE of the Government facilities and property, and the personnel necessary to conduct the work. Neither BATTELLE, nor the Government, nor DOE, nor their officers, employees, or authorized representatives shall be liable for any loss sustained by the SPONSOR, or others, where such loss is directly or indirectly attributable to the lawful exercise of the Government's right of control over health and safety at the Laboratory, or to suspension or cessation of activities under this agreement at the request of the Government pursuant to the Use Permit. The following applicable provisions of the Use Permit must be incorporated into any contract resulting from this proposal.

"8. PATENTS

A. (1) With respect to any invention or discovery conceived or first actually reduced to practice after September 30, 1979, by BATTELLE or its employees or consultants or by the employees or consultants of organizations sponsoring work at BATTELLE in the course of or under any agreement or activity undertaken pursuant to this Use Permit in which scientific or technical information and data developed by BATTELLE in the performance of work for DOE under the Operating Contract, but not reported to DOE on an unrestricted basis, (hereinafter referred to as "DOE Information and Data"), were specifically necessary for the conception or first actual reduction to practice of any invention or discovery, the Government, as represented by DOE, reserves a nonexclusive, irrevocable, royalty-free license for U. S. governmental purposes in any such invention or discovery and any patent application or patent that may result; provided, however, that nothing herein shall be deemed to modify or alter DOE's rights with respect to any invention or discovery made or conceived in the course of or under the Operating Contract. For purposes of this Article, scientific or technical information and data which are obtained by BATTELLE other than through its performance of the Operating Contract, or which are published, shall not be deemed to be "DOE Information and Data." To the extent necessary to enable DOE to determine

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the Government's rights under this Article, BATTELLE shall afford DOE the right to inspect the original records of inventions conceived or first actually reduced to practice after September 30, 1979, in the course of or under any arrangement or activity undertaken pursuant to this Use Permit; provided that no original records pertaining to work sponsored by third parties (other than BATTELLE affiliates or subsidiaries) shall be made available without the SPONSOR's consent which will not be unreasonably withheld, but if withheld then arrangements shall be made between counsel for BATTELLE and DOE for a review, at BATTELLE facilities, of the original record of any such invention and directly related information, with such records treated as proprietary data in accordance with the provisions of Article 10 for purposes of this review.

(2) BATTELLE shall report any invention or discovery covered by A (1) above within six months after conception or first actual reduction to practice and will advise DOE, within six months of such reporting, as to whether BATTELLE or the SPONSOR intends to file a United States patent application with respect to such invention or discovery; provided, however, if BATTELLE or the SPONSOR advises that neither desires to file, DOE may file any patent application, domestic or foreign, as it determines appropriate. If BATTELLE or the SPONSOR does not file a patent application within one year of reporting such invention or discovery, DOE, if it desires to file any patent application, will so notify BATTELLE and if BATTELLE or the SPONSOR does not file a patent application within 60 days after date of such notification, DOE may file any patent application, domestic or foreign, as it determines appropriate. If DOE files a patent application, all right, title and interest in and to such invention or discovery shall be assigned to DOE, subject to retention by BATTELLE or the SPONSOR, as the case may be, of at least a nonexclusive, irrevocable, royalty-free license for its own uses. With respect to any such filing, BATTELLE agrees that it will execute all documents and do all things necessary and proper to assist DOE and will use its best efforts to secure from its employees, the SPONSOR, and the inventor or inventors the execution of all documents and to have them do all things necessary and proper to assist DOE.

B. No claim for pecuniary award or compensation under the provisions of the Atomic Energy Act of 1954, as amended, shall be asserted by BATTELLE or its employees and consultants and employees and consultants of the SPONSOR with respect to any invention or discovery based on said "DOE Information and Data" to the extent described in A (1) above in the course of or under any arrangement or activity undertaken pursuant to this Use Permit.

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C. Except as otherwise authorized in writing by DOE, BATTELLE will obtain patent agreements to effectuate the provisions of paragraphs A and B of this Article from all BATTELLE employees or consultants, or employees or consultants of SPONSORS who perform any part of the work under this Use Permit while assigned to work at the Government-owned facilities, or while utilizing the Government-owned property or "DOE Information and Data," except nontechnical personnel, such as clerical employees and manual laborers.

9. ACCESS AND TECHNICAL DATA

A. To the extent necessary to fulfill the responsibilities of DOE under any law or regulation, or for the purposes of determining compliance with the terms of this Use Permit, DOE, at all reasonable times, subject to Article 10, shall have access to all or any part of the Government-owned facilities and shall have the right to inspect all activities conducted therein; provided, however, DOE's right to inspect shall not include the right to obtain technical data except as hereinafter provided.

B. The Government shall have the right to use for Governmental purposes any information or knowledge contained in any drawings, sketches, designs, data, specifications, technical and scientific data, photographs, negatives, reports, findings, recommendations, and memoranda of every description (all of which are hereinafter included in the term "technical data") made in the course of or under any arrangement or activity undertaken hereunder which specifically results from the use of said "DOE Information and Data."

C. BATTELLE shall furnish to DOE, without charge, one copy of any technical data as to which the Government has rights under B above. To the extent necessary to enable DOE to determine the Government's rights under this Article, DOE shall have the right to inspect the technical data specifically resulting from the use of said "DOE Information and Data" which is made in the course of or under any arrangement or activity undertaken hereunder, provided that no proprietary information of a SPONSOR (other than BATTELLE subsidiaries and affiliates) shall be made available without the SPONSOR's consent, which will not be unreasonably withheld.

D. When furnishing any technical data referred to in Paragraph C above, BATTELLE or the SPONSOR, as the case may be, will make reasonable efforts to identify any invention made by BATTELLE or the SPONSOR if not previously reported.

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10. PROPRIETARY INFORMATION

Except for the rights to use provided in Article 8 A and Article 9 B, DOE shall exert its best efforts to (1) protect information obtained pursuant to this Use Permit as proprietary and not use such information in any way which will violate its proprietary nature; (2) communicate, disclose or publish any part of such information within the Government only to the extent such communication, disclosure or publication is required in the administration of this Use Permit; (3) insure against communication, disclosure or publication outside the Government of any part of such information, without the written consent of BATTELLE, except to the extent such information is (a) generally known or available from other sources without obligation concerning its confidentiality, (b) available to others without obligation concerning its confidentiality, or (c) available to the Government without obligation concerning its confidentiality; provided nothing herein shall be deemed to limit or restrict the right of DOE to communicate, duplicate, use or disclose such information as required to fulfill the responsibilities of DOE pursuant to any law including applicable Executive Orders and Federal Regulations."

SOLE ACCOUNT OF BATTELLE. This agreement is not entered into by BATTELLE for the account of the Government or DOE but is for the sole account of BATTELLE.

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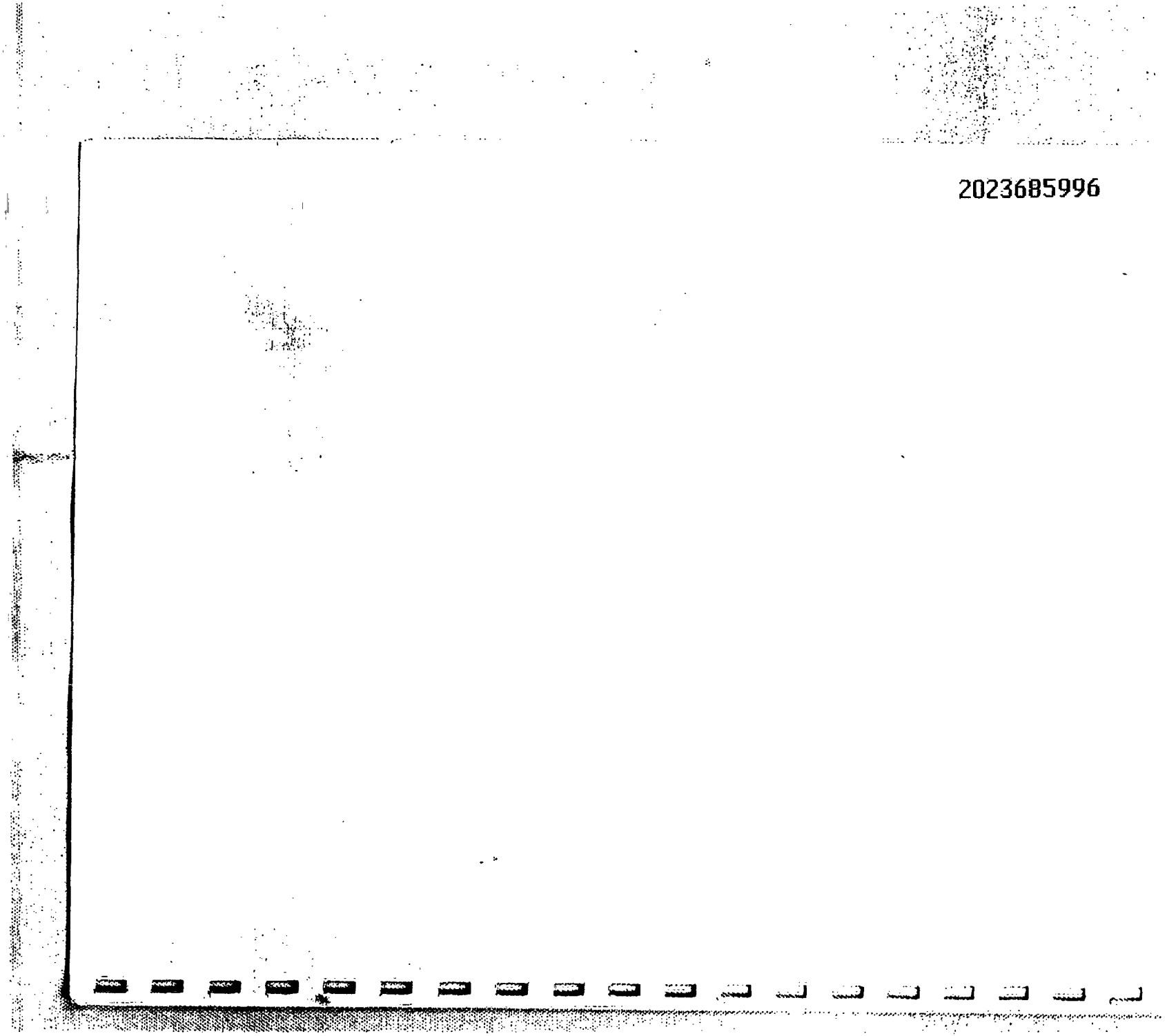

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